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A SOLUBLE PYRIDINE NUCLEOTIDE OXIDASE SYSTEM FROM BARLEY ROOTS [1]

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The changing response to certain respiratory inhibitors during the development of barley (*Hordeum vulgare* L.) roots suggests the gradual disappearance of cytochrome oxidase (14, 15, 18), yet its continued presence has been demonstrated by extraction and assay (8, 11, 12). This brings up an intriguing problem concerning the changes in terminal oxidase pattern during root ontogeny. However, before the details of the apparent ontogenetic change can be elucidated, it seems necessary to study more thoroughly the complement of active or potentially active oxidases existing in the roots, and to identify the cofactors necessary for their operation. It is conceivable that the apparent ontogenetic changes are reflections of changes in concentrations of substances affecting the activities of the various oxidase systems and that actual changes in the concentrations of the enzyme proteins themselves are of lesser importance. Evidence is presented here for the existence of a highly active, non-cytochrome, pyridine nucleotide oxidase system in homogenates of barley roots. This oxidase is inhibited by *ortho*-dihydric phenols and accelerated by hordenine and tyramine. The latter naturally occurring monohydric phenols are known to undergo marked changes in concentration during development of barley roots (26).

MATERIALS AND METHODS

All roots used in this study were grown from the same lot of Atlas 46 barley seed from the 1955 crop. The seed was stored at 0° to 3° C until used for the experiments, which were performed in 1956 and 1957.

The apparatus in which the roots were grown (fig 1) was originally designed to permit production of straight, uniform roots having sparse root hairs and suitable for experiments in which large numbers of serial segments had to be prepared. A novel feature was an internal, but isolated, aeration and circulation system which in our experience avoided the curling and dwarfing which resulted from direct impingement of streams of bubbles from aerators upon growing roots. Provision was also included for gradually supplanting the ambient solution with fresh volumes thereof without necessitating removal of roots from it. This took the place of solution replacement as practiced by Epstein and Hagen (5) in their procedure for growing barley roots.

The equipment was installed in a darkroom in which the temperature was controlled to maintain a solution temperature of 20° ± 1° C in the apparatus.

The basic component of each unit of the apparatus was a tank 9 inches deep, 16 inches long, and 12 inches wide fabricated from stainless steel. When filled to the operating level, about 1 inch from the top, the capacity of such a tank is about 26 liters.

Seeds or seedlings were supported over the tank

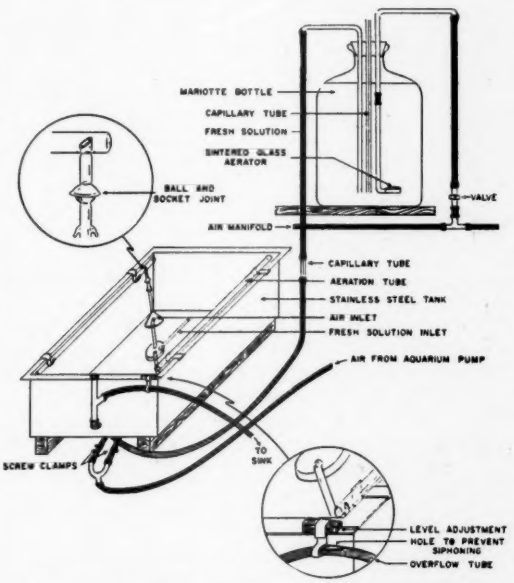

FIG. 1. Schematic diagram of one unit of the apparatus used for growing barley roots. A perforated tray used to support seedlings over the tank is not shown.

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by means of a perforated screen tray. The material was stainless steel sheet in which holes of 0.140 inch diameter were punched. Holes were arranged in staggered rows so as to yield 51 % open space.

To guard against the possibility of metal ions leaching from the steel, both tanks and trays were given 2 spray coatings of a transparent acrylic resin (Krylon Crystal-Clear Spray Coating, made by Krylon Inc., Norristown, Pa.) before use. Autoclaving after spraying assured removal of solvents. Coating and autoclaving were repeated when imperfections developed in the resin layer.

The ambient solution in the tank was distilled water which had been made 7.5×10^{-5} M in calcium sulfate and 2.5×10^{-5} M in magnesium sulfate. Although barley roots can readily be grown in 10^{-4} M calcium sulfate alone (5), replacement of one fourth of this amount of Ca^{++} with Mg^{++} seemed to enhance slightly the uniformity and rate of growth of roots. The solution was continuously aerated and slowly circulated while it was also being gradually supplanted by fresh solution. Air was supplied by a 2-watt vibratory aquarium pump and was allowed to bubble from delivery tubes into the solution under inverted funnels in opposite corners of the tank. The bubbles rising through the stems of the funnels carried solution with them, beginning the aerating process while also acting as a pump to circulate the solution. The funnels were connected to the horizontal aeration tubes (i.d. about 1 cm) which resulted in the movement of trains of bubbles through the latter. The aeration tubes were supported a few millimeters above the surface of the solution. Holes (1 mm in diameter) in the bottom walls of the tubes allowed the aerated solution to drip out while excess air escaped through the open far ends of the tubes. Bubble size and rate of aeration and circulation were controlled by slightly raising or lowering the free ends of the aeration tubes and by adjusting the air flow rate.

Connected to each tank was a 14-liter Mariotte bottle filled with fresh solution. Air was bubbled through this solution for at least 30 minutes before delivery into the tank began. The solution was then fed into the bottom of the tank at the end opposite the overflow at the rate of about 1 liter per hour. The overflow was adjusted to maintain the solution level 3 to 5 mm below the bottom of the tray. This solution supplanting process was begun as soon as the roots had grown down into the solution and was continued at the rate of 14 liters per day until the roots were harvested. Failure to do this for several days resulted in increased root hair development and crooked root growth even when normal aeration was continued.

Seed to be germinated, in lots of 30 g, was soaked in continuously aerated distilled water for 24 hours at 20° C. The wet seeds were then transferred to a perforated tray, spread out uniformly, and covered with a layer of paper towels saturated with solution from the tank. Saturation was maintained for 48 hours by means of paper towel wicks, which were then removed. The growing seedlings raised the layer of towels which was also discarded when it had become dry. Age of the roots was counted from the time the seed was first wetted.

When barley roots were grown by the method described, fungal contamination was not a problem. Tanks, trays, and glassware, however, were autoclaved between crops as an extra precaution.

Roots were generally harvested when 6 days old, at which time they were about 10 cm long. The excised roots were quickly blotted with paper towels, weighed, wrapped in waxed paper, and chilled in a cold room for 20 to 30 minutes. All subsequent operations in the preparation and fractionation of homogenates were performed at 0° to 3° C.

The chilled roots were ground in a mortar for 2.5 minutes with 0.5 g of reagent grade sand and 3 ml of 0.02 M potassium phosphate buffer (pH 7.4) per gram of sample. In some early experiments the buffer was made 0.5 M in sucrose, but it was soon found that tonicity control was not necessary for the demonstration of pyridine nucleotide oxidase activity. The homogenate was centrifuged at $500 \times G$ for 10 minutes. The supernatant liquid was decanted and centrifuged at $27{,}000 \times G$ for 20 minutes, yielding a second supernatant fraction. This was the crude enzyme preparation used in most experiments, but in a few experiments it was subjected to further centrifugation at $144{,}000 \times G$ for 20 minutes.

All enzyme assays were performed at 30° C, either in a Warburg apparatus or in a Beckman DU quartz spectrophotometer having a controlled temperature cell compartment.

Reduced pyridine nucleotide oxidase activity was routinely assayed by a spectrophotometric method based on the disappearance of the absorption band centered at 340 mμ upon oxidation of the reduced nucleotides. Three matched cuvettes of 1 cm light path were used. The test cuvette received 0.1 ml of enzyme preparation containing approximately 40 μg of nitrogen, 0.2 ml of 1.15×10^{-3} M reduced pyridine nucleotide, 1.5 ml of 2×10^{-2} M potassium phosphate buffer at pH 6.4, and water to make a total volume of 3 ml. The initial optical density at 340 mμ was approximately 0.5. The reference cuvette was complete except for nucleotide. The control cuvette was identical to the test cuvette except that it received boiled instead of active enzyme preparation. The reaction was started by adding enzyme to the test cuvette, then covering and twice inverting it. Optical density readings were taken after 1 minute and at successive 1-minute intervals for at least 8 minutes. During that time the optical density of the test cuvette in typical assays decreased by about 0.25 while that of the control cuvette decreased by only 0.001 to 0.005.

For the assay of reduced pyridine nucleotide oxidase activity after dialysis some changes were made in the above procedure. Essential components lost during dialysis were replaced by adding 0.1 ml of a boiling water extract of roots. This volume contained the extracted materials from 1 g fresh weight of 6-day-old roots. Because of the greatly enhanced ac-

tivities elicited by root extracts, the enzyme preparations were diluted 10- to 50-fold before assay.

The rate of decrease of optical density at 340 mμ was a measure of the rate of oxidation of the nucleotide. The data were converted into microliters of O_2 consumed per hour per milligram of total nitrogen in the enzyme preparation ($Qo_2(N)$). This conversion was accomplished by application of an equation[4] derived from Beer's law and the molecular extinction coefficient of the reduced pyridine nucleotides. The latter was taken to be 6.22×10^6 cm² per mole (13). It was first assumed and later demonstrated that oxidation of 1 micromole (μM) of DPNH (reduced diphosphopyridine nucleotide) or TPNH (reduced triphosphopyridine nucleotide) by this system is accompanied by the consumption of 11.2 μl (0.5 μM) of O_2.

The manometric assay of DPNH oxidase differed from the spectrophotometric assay in that the initial substrate concentration was much higher and a larger aliquot of enzyme preparation was used. The Warburg vessels contained 0.1 to 0.5 ml of enzyme and sufficient water and pH 6.4 potassium phosphate buffer to make the buffer concentration of the 3 ml reaction mixture 0.01 M. After 10 minutes equilibration, the reaction was started by tipping in the substrate solution from a sidearm. Usually 4 mg of $Na_2DPNH \cdot 4\ H_2O$ was used per flask. Two control flasks were included; one in which boiled enzyme preparation was substituted for fresh, and another having active enzyme but lacking substrate. Center wells contained filter paper squares saturated with 10 % NaOH solution.

Loss of DPNH oxidase activity of enzyme preparations stored at 0° C was 5 to 10 % for the first 24 hours. By storing at −20° C it was possible to preserve preparations for several months without appreciable loss of activity. Hence numerous tests could be made using enzyme from the same source.

Cytochrome oxidase activity was measured by the spectrophotometric method of Cooperstein and Lazarow (4) as modified by Fritz and Beevers (8). DPNH cytochrome c reductase was estimated by the method of Evans (7).

The total nitrogen content of the various preparations was estimated by a method based upon that of Miller and Miller (24) but differing from it in that the entire procedure was performed in Taylor digestion tubes and that an air stream was used to mix the solutions.

DPNH and TPNH were obtained from Sigma Chemical Co. Crystalline hordenine sulfate and tyramine hydrochloride were purchased from Mann Research Laboratories, Inc. The authenticity of the latter compounds was verified by suppliers assay, by ultraviolet absorption spectra, and by melting point determinations.

[4] For a 1 cm light path and 3 ml reaction volume and when $Qo_2(N) = \mu l$ of O_2 consumed/hr/mg of N, the following relations can be shown to hold:

$$Qo_2(N) = \frac{\Delta \text{ optical density/min} \times 60 \times 3 \times 22.4 \times 0.5}{6.22 \times \text{mg N}}$$

$$Qo_2(N) = \frac{\Delta \text{ optical density/min}}{\text{mg N}} \times 324$$

Results

Demonstration of DPNH Oxidase Activity: Crude enzyme preparations derived from barley roots, by clarifying homogenates at 500 × G for 10 minutes and then subjecting the supernatant fraction to a second centrifugation at 27,000 × G for 20 minutes, catalyze the rapid oxidation of reduced pyridine nucleotides. In a typical experiment the enzyme was derived from 6-day-old roots, and 0.1 ml, containing about 40 μg of nitrogen, was used per cuvette. The corrected $Qo_2(N)$ values shown by the first 5 minutes of readings were 240 and 390 for the oxidation of DPNH and TPNH respectively. Similar results were obtained with numerous other preparations from roots of 5- to 7-day-old plants. At this stage of the investigation there was no cofactor requirement, and DPNH disappearance in the presence of 0.1 ml of enzyme preparation was adopted as the standard assay for preliminary characterization of the system.

Although DPNH oxidase activity could be demonstrated throughout a wide range of pH values, routine tests were run at pH 6.4 where optimal activity was obtained.

Spectrophotometric assays for DPNH oxidase measured only the disappearance of DPNH. Proof that this disappearance was accompanied by O_2 consumption was supplied by manometric assays using substrate amounts of DPNH. The initial concentration of DPNH in the Warburg flasks was 1.55×10^{-3} M as compared to only 7.67×10^{-5} M in the spectrophotometer cuvettes. Results of a manometric assay in which 0.15 ml of supernatant enzyme from 6-day-old roots was used to catalyze the oxidation of 4 mg of $Na_2DPNH \cdot 4\ H_2O$ (90.5 % pure), indicated a final O_2 uptake of approximately 94 % of the theoretical calculated for the oxidation of the DPNH to DPN^+ and water (kinetics similar to control curve, fig 3). Non-enzymatic oxidation of DPNH was negligible. The $Qo_2(N)$ value for the period from 5 to 15 minutes was about 1500. Comparable values were obtained in other manometric assays. In 9 assays the total O_2 uptake varied from 90 to 112 % of the theoretical, averaging 101 %.

Additional evidence that the action of the enzymes in the supernatant upon DPNH is a true oxidation was gained by studying the ultraviolet absorption spectra of the spectrophotometric and manometric assay mixtures. When the absorption spectrum obtained before addition of the enzyme preparation was compared with that of the mixture after the reaction had stopped, it was evident that the effect of enzyme action was to eliminate the absorption band at 340 mμ and to increase slightly the intensity of the 260 mμ absorption band. This is in accord with oxidation of DPNH to DPN^+.

TABLE I

EFFECT OF CENTRIFUGATION OF A BARLEY ROOT HOMOGENATE UPON THE DPNH OXIDASE ACTIVITY OF THE SUPERNATANT FRACTION

SUPERNATANT	CENTRIFUGATION MAX FORCE G	CENTRIFUGATION TIME MINUTES	μG N PER 0.1 ML	DPNH OXIDASE ACTIVITY* $Qo_2(N)$
I	500	10	45	210
II	27,000	10	41	205
III	144,000	20	32	280

*Activity was calculated on the basis of decrease in optical density at 340 mμ during the period from 1 minute to 6 minutes after the reaction was started.

It was not possible to bring about any appreciable decrease in DPNH oxidase activity by centrifugation of root homogenates (table I). Removal of particles of the mitochondrial size range from Supernatant I was accompanied by little loss of activity. Supernatant II, however, was still slightly turbid and when examined by dark field microscopy it was found to contain countless particles visible only as points of light. Removal of these particles yielded the completely clear Supernatant III in which no particulate matter was resolvable by dark field microscopy. It is obvious that most of the oxidase activity remained in the final supernatant and that the nitrogenous materials associated with the particulate fractions were largely inactive. It may be concluded that the particulate components of the cytoplasm are not necessary for the in vitro functioning of the DPNH oxidase system, but that their presence does not interfere with its activity. The system may be referred to as "soluble" in the sense that the enzymatic proteins are not sedimented from aqueous solution by centrifuging at 144,000 $\times$ G for 20 minutes. However, the possibility has not been eliminated that the oxidase system as it exists in the intact cell may be associated with easily disrupted cytoplasmic particles (28).

Measurable cytochrome oxidase and DPNH cytochrome c reductase activities were found in the DPNH oxidase preparations. Such activities, however, were only a few percent of those which would be necessary to mediate the electron transport from DPNH to O_2 if cytochrome c and cytochrome oxidase were major components of the oxidase system.

EFFECT OF ROOT EXTRACT: The standard assay of the DPNH oxidase system employed only enzyme, substrate, and buffer. It was found, however, that addition of an extract prepared by boiling macerated barley roots (6 days old) in water for 10 minutes and concentrating so that 1 ml contained the yield from 10 g of roots, caused a great increase in the rate of enzymatic oxidation of DPNH. This effect was apparent when 0.1 ml of extract was used with 0.1 ml of enzyme preparation, but was much more pronounced when less enzyme was used (table II). Thus when 5 μl of supernatant was assayed in the presence of 0.1 ml of the extract, the specific activity was 18.5 times that obtained by the standard assay using 100 μl of supernatant. Even this was not maximal as was shown by a further increase when 0.2 ml of the extract was used. No attempt was made to attain truly maximal activities.

One explanation for the great stimulatory effect of the root extract is that it supplies additional amounts of heat-stable cofactors which limit the reaction rate in the standard assay. When it is considered that the cytoplasmic constituents were diluted perhaps 20-fold during homogenization and an additional 30-fold when 0.1 ml was used for assay, it is apparent that any native cofactors would be present in far lower concentration in the assay cuvette than in the intact root cell. The accelerating effect of the extract might arise from the counteraction of this dilution of cofactors. Such a concept is in agreement with numerous observations that the absolute DPNH oxidase activity per cuvette was not a linear function of the amount of enzyme used. For example, the activity obtained when 0.05 ml of supernatant was assayed was always less than half of that obtained with 0.1 ml, and the effect became more pronounced when lesser volumes were used.

Dialysis of the concentrated root extract against distilled water for 40 hours resulted in the loss of nearly all of its cofactor activity. A concentrate of the external solution containing the dialyzable components of the extract had all the initial activity. This supports the idea that root extract contains dialyzable, heat-stable substances which may be essential to the operation of the DPNH oxidase system.

When compared on a dry weight basis, boiled solutions of commercial malt extract had only a small fraction of the cofactor activity of root extract and, when mixed with root extract, had a considerable

TABLE II

EFFECT OF CONCENTRATED ROOT EXTRACT UPON DPNH OXIDASE ACTIVITY OF CRUDE SUPERNATANT ENZYME PREPARATIONS FROM 6-DAY-OLD BARLEY ROOTS

VOLUME OF SUPERNATANT PER CUVETTE μl	VOLUME OF CONCENTRATED ROOT EXTRACT ADDED PER CUVETTE ML	DPNH OXIDASE ACTIVITY $Qo_2(N)$	DPNH OXIDASE ACTIVITY AS % OF STANDARD ASSAY * VOLUME BASIS
100	none	205	100
100	0.10	260	127
25	0.10	1270	910
10	0.10	2430	1180
5	0.10	3800	1850
5	0.20	4400	2140

* In the standard assay 100 μl of enzyme preparation in a total volume of 3.0 ml per cuvette was allowed to act upon DPNH at an initial concentration of 7.7×10^{-5} M. No root extract or other cofactor source was added. The first line of values pertain to this standard assay of preparation.

inhibitory effect upon the activity of the latter. This was true also of boiling water extracts of barley seeds. The basis of these inhibitions was not investigated.

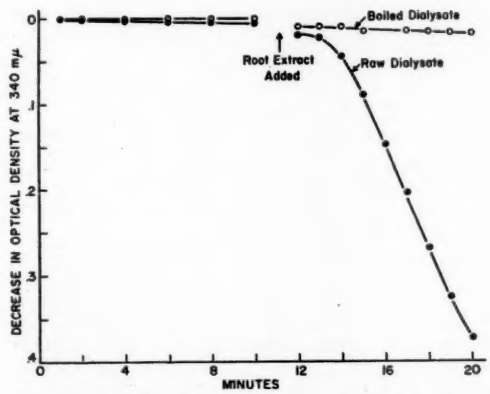
DIALYSIS AND REACTIVATION OF DIALYZED ENZYME: The possible existence of dialyzable cofactors of the DPNH oxidase system was further investigated by studying the effect of dialysis upon enzyme preparations. Preparations to be dialyzed were first treated in 50 ml lots with 2 ml of 2.5 % protamine sulfate solution at pH 7.0 (*29*) and then briefly exposed to pH 4.5 (acidified with HCl then immediately neutralized with KOH). A clear solution was obtained by removing the resulting precipitate by centrifugation. Loss of oxidase activity as a result of these treatments was 10 to 20 %.

Dialysis at 0° C against 0.01 M potassium phosphate buffer (pH 7.0) was accompanied by a rapid loss of oxidase activity. This amounted to about 50 % in the first 2 hours. After 40 hours the remaining activity was only about 4 % of the undialyzed control. During the period in which the major part of the activity was lost, the rate of loss was a linear function of the logarithm of the elapsed time, as would be expected if the loss of activity were a result of outward diffusion of a rate limiting cofactor.

After inactivation by dialysis, the DPNH oxidase system can be reactivated by the addition of barley root extract. When 0.05 ml of the extract was added to the assay mixture containing 0.1 ml of a preparation dialyzed for 40 hours, maximum reactivation was attained in about 3 minutes (fig 2). Due to losses incurred during treatment with protamine sulfate and acid and through aging during dialysis, the absolute DPNH oxidase activity per unit volume of reactivated enzyme was always less than that of the untreated supernatant. However, because about 80 % of the total nitrogen had been removed by these procedures, the specific activities of the reactivated preparations were higher than the initial values for the crude preparations. Thus a typical crude enzyme preparation from 6-day-old roots had a $Qo_2(N)$ of 1980 when a 5 µl aliquot was assayed in the presence of 0.1 ml of root extract, whereas the values obtained with a typical reactivated 40 hour preparation varied from 2400 to 6000, depending upon the volume of extract and enzyme used in the assay. In earlier experiments employing the standard DPNH oxidase assay (0.1 ml of enzyme preparation and no added cofactors or extract), similar preparations had yielded Qo_2 (N) values of only 200 to 300. The much higher values given by the reactivated preparations were due partly to the accelerative effects of root extracts and partly to the removal of inactive nitrogenous components or inhibitors by the purification procedures.

None of the materials tested as possible cofactor sources (e.g. yeast extract, liver coenzyme concentrate, and a solution of root ash) could replace the root extract in reactivating dialyzed enzyme, and all of them had inhibitory effects upon the reactivation elicited by root extract. Because of this it seemed unprofitable to search for the active cofactors in any material other than barley roots.

NATURE OF COFACTORS: Suggestions of the nature of some of the cofactors removed from enzyme preparations by dialysis and restored by boiled root extract were obtained from 2 sources. Firstly, passage of the active, dialyzable fraction of root extract through a column of Amberlite ion exchange resin IRA-400 in the carbonate form removed most of the cofactor activity. Elution of the column with a 4 %



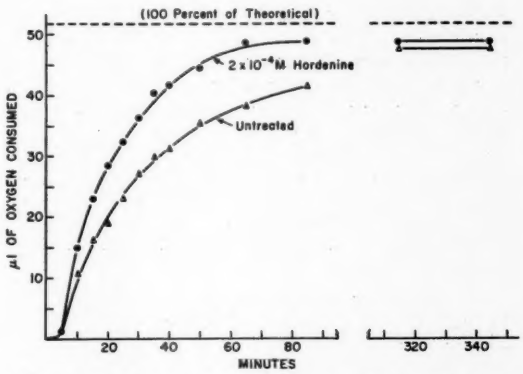


FIG. 2 (*left*). Reactivation of a DPNH oxidase preparation after 40 hours of dialysis by the addition of a boiling water extract of roots. Both the enzyme preparation and the extract were derived from 6-day-old barley roots.

FIG. 3 (*right*). The effect of hordenine upon the rate of oxygen uptake during the oxidation of DPNH by a supernatant enzyme preparation from 6-day-old barley roots. Four milligrams of substrate (90.5 % $Na_2DPNH \cdot 4\,H_2O$) and 0.15 ml of enzyme preparation were used in each Warburg flask. The pH was controlled at 6.4 with 0.01 M potassium phosphate buffer. The total oxygen uptake was about 94 % of the theoretical both with and without hordenine. Autooxidation was negligible.

ammonium bicarbonate solution produced a turbid eluate. The latter was cleared by centrifugation and excess ammonium bicarbonate was removed from the supernatant by vacuum evaporation. The water-insoluble solid fraction appeared to be an amphoteric substance capable of forming an easily soluble hydrochloride. Neither the supernatant fraction, nor the hydrochloride of the insoluble fraction, had much cofactor activity, but when the 2 were combined they were highly effective in reactivating exhaustively dialyzed enzyme preparations. Further tests suggested that the insoluble component might be alkaloidal in nature. Secondly, a series of early experiments with tyrosine and related compounds had shown that the DPNH oxidase system was slightly inhibited by alanine and phenylalanine, slightly accelerated by tyrosine, and accelerated several fold by diiodotyrosine and thyroxine. Thus it seemed reasonable to investigate alkaloidal derivatives of tyrosine as possible cofactors of the oxidase system.

Young barley roots have long been known to contain tyrosine derivatives in the form of the phenolic alkaloidal amines, hordenine and tyramine, in appreciable amounts, although these compounds are lacking in the seeds (26). Hence these alkaloidal amines were included in a series of compounds tested for their inhibitory or acceleratory effects upon the DPNH oxidase system. Hordenine and tyramine were also tested for their ability to reactivate exhaustively dialyzed enzyme preparations, but were found to have an effect only when some root extract also was added.

INHIBITOR AND ACCELERATOR STUDIES: The effect upon the DPNH oxidase system of each of the various compounds tested was determined by comparing the activity of an aliquot of a crude supernatant enzyme preparation in the presence of a 10^{-4} M concentration of the compound to the activity of an untreated control aliquot. Aliquots of the same enzyme preparation from 6-day-old roots were used for the entire study. The enzyme and test compound were incubated together for 5 minutes before DPNH was added to start the reaction. The rate of oxidation was determined by measuring the decrease in optical density at 340 mμ during the period 1 minute to 6 minutes after addition of DPNH. Each compound was tested at least twice, and the mean of the results was compared to the mean of at least 2 assays of the untreated control. The results of each assay were corrected for a slow non-enzymatic oxidation of DPNH.

It was found that the DPNH oxidase system is highly sensitive to numerous phenols, to cyanide, to ascorbic acid, and to several metal chelating agents. In summary, the results (table III) indicate that, (a) the *para*-monohydric phenols, 3,5-diiodotyrosine, tyramine, and hordenine are effective accelerators, (b) all the *ortho*-dihydric phenols tested are potent inhibitors, (c) also inhibitory are hydroquinone, cyanide, ascorbic acid, diethyldithiocarbamate, 1,10-(*ortho*)-phenanthroline, and 2,2′-dipyridyl. It is interesting to note that β-phenylethylamine and ephedrine, non-phenolic analogs of tyramine, are relatively inactive, as is phenylalanine, the non-phenolic analog of tyrosine. These data, taken collectively, emphasize the importance of the *para*-monohydric phenol structure, the relative inactivity of non-phenolic analogs, and the potent inhibitory qualities of the *ortho*-dihydric phenol structure. The greater increase in activity elicited by hordenine and tyramine in comparison with phenol and tyrosine indicates that the alkyl amine side chain is functional in rendering these compounds more active.

TABLE III

EFFECT OF VARIOUS COMPOUNDS UPON DPNH OXIDASE ACTIVITY OF SUPERNATANTS DERIVED FROM 6-DAY-OLD BARLEY ROOTS

COMPOUND *	EFFECT	
	ACCELERATION (%)	INHIBITION (%)
Phenol	35	
Resorcinol	3	
Hydroquinone		92
Catechol		98
o-Cresol	11	
DL-Phenylalanine		1
DL-Dihydroxyphenylalanine		83
L-Tyrosine	11	
3,5-Diiodo-L-tyrosine**	77	
Tyramine **	65	
Hordenine **	54	
β-Phenylethylamine	4	
Ephedrine	14	
L-Epinephrine		81
L-Arterenol		97
N-Isopropyl-L-arterenol		92
Protocatechuic acid		97
Caffeic acid		96
3,4,5-Trihydroxybenzoic acid		81
p-Hydroxybenzoic acid	7	
2,4-Dinitrophenol		8
Phenylthiourea		20
1,10-(*ortho*)-phenanthroline		69
2,2′-Dipyridyl		69
Diethyldithiocarbamate		98
Cyanide		92
Cysteine		18
Ascorbic acid		93

* All compounds were tested at 1×10^{-4} M.

** Effects several-fold greater than this could be obtained by using less enzyme preparation in the assay.

The basis of the inhibition caused by the *ortho*-dihydric phenols and hydroquinone has not yet been investigated experimentally, but it seems unlikely that these compounds act merely as reductants, because the oxidation of catechol and hydroquinone could not be demonstrated under the conditions employed for the manometric assay of the oxidase system. There was likewise no measurable O_2 uptake when 0.3 ml aliquots of 0.05 M hordenine and tyramine were incubated with 2.7 ml of active enzyme preparation in the Warburg apparatus for 1 hour.

The acceleratory effects of hordenine and tyramine were confirmed by several experiments on different batches of enzyme prepared from 6-day-old

roots, and it was found that the percentage of increase could be greatly enhanced by merely decreasing the volume of enzyme used in the assay (increasing the dilution of native cofactors or inhibitors). This effect was analogous to that obtained with boiled root extract (table II).

An increased rate of O_2 consumption accompanied the increased rate of DPNH disappearance elicited by horenine. In a typical manometric experiment, the oxidation of 4 mg of $Na_2DPNH \cdot 4\ H_2O$ (90.5 % pure) was catalyzed by 0.15 ml of enzyme preparation in a final reaction volume of 3 ml. One test flask contained hordenine at a final concentration of 2×10^{-4} M and the other was untreated. Appropriate controls also were included. The results (fig 3), in agreement with spectrophotometric data, indicated an increased rate of DPNH oxidation in the presence of hordenine. The total O_2 uptake with and without hordenine was, respectively, 94 and 93 % of the theoretical total for the complete oxidation of the supplied DPNH to DPN^+. Several other manometric experiments also yielded data showing that hordenine increased the initial rate of O_2 uptake without changing the final mole ratio of O_2 consumed to substrate oxidized.

Extraction of Alkaloidal Amines from Barley Roots: The idea that tyramine and its methylated derivative, hordenine, might be natural cofactors of the soluble DPNH oxidase system is especially attractive because hordenine has long been known to occur in barley roots (23, 25, 26). More recent work (6, 19, 20, 21, 22) has shown that *N*-methyltyramine accompanies hordenine in many varieties of barley, and that in some it appears to replace hordenine completely (19). Tyramine has been found in only a few varieties (6). To the writer's knowledge the complement of alkaloidal amines present in roots of Atlas 46 barley had not been studied previously. A preliminary study was therefore undertaken.

Roots were extracted by the procedure of Kirkwood and Marion (19). The resulting crude extract, along with pure tyramine and hordenine, was subjected to paper chromatography, the technique used being identical to that of Leete et al (21), except that descending solvent flow was used. When the papers were sprayed with Millon's reagent, red spots revealed the locations of phenolic compounds, in this case presumably tyramine and its methylated derivatives. The extract alone or mixed with known hordenine and tyramine yielded 3 well-defined spots positive to Millon's test. These were referred to as M_1, M_2 and M_3 in order of their increasing distance from the origin. The R_f values of the spots on replicate chromatograms were determined and the mean values were compared for extract, hordenine, and tyramine individually, and mixtures of extract with hordenine and tyramine. From these data it was concluded that M_1 and M_3 were tyramine and hordenine respectively. This seemed justified in view of the extensive work by Kirkwood and Marion (19, 20) and Leete et al (21) which demonstrated that the methods of extraction and chromatographic separation employed by them and used in this study were specific for tyramine and its methylated derivatives. These investigators also isolated from barley roots a compound which had an R_f similar to M_2 and were able to identify it as *N*-methyltyramine. M_2 and M_3 were of approximately equal intensities, whereas M_1 was much weaker, suggesting that the methylated derivatives of tyramine were more plentiful in the extract than was the amine itself. By means of the spot area method it was estimated that 100 g of fresh 6-day-old roots yielded roughly 10 mg each of *N*-methyltyramine and hordenine.

Discussion

Mitochondrial particles obtained from barley roots contain cytochrome oxidase (12, 27). This oxidase is probably functional in the oxidation of DPNH generated within the mitochondrial body. It is possible that some DPNH produced by non-mitochondrial systems may also be oxidized by this pathway. In barley root homogenates, however, most added DPNH is oxidized by a system which is not dependent upon mitochondrial enzymes. This is obvious from the results of experiments in which removal of cytoplasmic particles from homogenates had little effect upon the absolute activity of the oxidase system and actually increased the specific activity (table I).

Results of several different types of experiments support the idea that the enzymes remaining in the supernatant after high-speed centrifugation of barley root homogenates constitute a true oxidase system. This system can transfer electrons from DPNH to O_2 without requiring the addition of unphysiological electron carriers. Because it has been shown (27) that these same supernatant enzyme preparations contain a mechanism capable of reducing DPN^+ to DPNH by the oxidation of malate, it is presumed that malate also can be oxidized through this oxidase system.

The number of components in the system cannot be determined from the present data. That more than a single cofactor is involved was indicated by the finding that the cofactor activity of fractioned root extracts could be reconstituted in any large measure only be recombining 2 of the fractions.

The experiments concerning localization, inhibition characteristics, and possible cofactor requirements of the oxidase system are insufficient to permit positive identification of its terminal component. However, extensive inhibition by cyanide, diethyldithiocarbamate, 1,10-(*ortho*)-phenanthroline, and 2,2'-dipyridyl indicates that a heavy metal is involved.

The typical particle-bound cytochrome oxidase can be eliminated because: (a) Cytochrome oxidase is known to be localized on cytoplasmic particles, yet removal of these particles from homogenates did not appreciably reduce DPNH oxidase activity. (b) The DPNH cytochrome c reductase and cytochrome oxidase activities of the preparations were far less

than those necessary to mediate the oxidation of DPNH at the observed rates. (c) The oxidase was almost totally inhibited by 10^{-4} M diethyldithiocarbamate, whereas according to James and Garton (17) the cytochrome oxidase of barley is relatively insensitive to this concentration of the compound.

Polyphenol oxidase can also be eliminated because: (a) This oxidase has not been found in roots of cereal seedlings although several investigators have looked for it (16, 30). (b) The barley roots used in this study also showed no polyphenol oxidase activity; the supernatant enzymes did not oxidize catechol and no colored product was formed from dihydroxyphenylalanine. (c) The system was inhibited by numerous *ortho*-dihydric phenols which are substrates of polyphenol oxidase (table III).

Ascorbic acid oxidase appears to be the terminal component of a soluble DPNH oxidase system extracted from potato tubers by Hackett (9, 10). Nevertheless, it seems unlikely that this enzyme is an essential part of the soluble DPNH oxidase system of barley roots because: (a) Like the peroxidase-linked TPNH oxidase system extracted from wheat germ meal by Conn et al (3), the barley root system is extensively inhibited by 10^{-4} M ascorbic acid. Such inhibition is not compatible with the concept of an electron transport system utilizing ascorbic acid and dehydroascorbic acid as a redox pair. (b) Manometric experiments showed that DPNH oxidase preparations oxidized ascorbic acid much less rapidly than DPNH.

Peroxidase is known to be present in barley root extracts (16). If peroxide were also present, peroxidase could function in the oxidation of phenols and other compounds. Although the sensitivity of the DPNH oxidase system to diethyldithiocarbamate suggests a copper-containing terminal oxidase (17), the appreciable inhibition caused by 1,10-(*ortho*)-phenanthroline and 2,2′-dipyridyl could be taken as evidence for the involvement of ferrous iron (2). Peroxidase is not usually considered to be a true terminal oxidase because it does not catalyze the reduction of O_2. Instead, it brings about the conversion of peroxide to water at the expense of an oxidizable substrate, and its continued operation depends upon the production of peroxide by another system. Such a peroxide producing system is not known to be present in barley root homogenates. James and Cragg (16) were unable to detect any peroxide in barley seedling preparations although they used several highly sensitive methods. Their preparations oxidized catechol only after addition of peroxide to the assay mixture. Enzyme preparations used in the present investigation also were unable to oxidize catechol. Furthermore, the addition of peroxide induced bubbling, suggesting the presence of active catalase. It is therefore questionable whether any effective concentration of peroxide could exist in the DPNH oxidase preparations. Aside from this difficulty, oxidation of DPNH to DPN^+ with the uptake of 0.5 mole of O_2 per mole of nucleotide appears not to be consistent with linkage to a typical peroxidase-dependent system.

Nevertheless, Akazawa and Conn (1) have shown that an artificial electron transport system consisting only of Mn^{++}, a monohydric phenol carrier (or resorcinol), and crystalline peroxidase can oxidize DPNH with the same stoichiometry as observed in the present experiments. I have found that hordenine or tyramine can fulfill the phenol requirement of this system, though less efficiently than resorcinol. Resorcinol, however, is inactive in the barley root system. Numerous differences in inhibitor characteristics suggest that the barley root oxidase is different from, and more complex than the system described by Akazawa and Conn, but the possibility remains that some type of peroxidase-oxidase system may be functional in the barley root preparations.

It is conceivable that the soluble DPNH oxidase system is activated only upon cellular disruption. Another possibility is that the system is operative in intact root cells and that it constitutes a bypass around the cytochrome system. If, as experiments indicate, hordenine and tyramine act as intermediates, whereas ascorbic acid and *ortho*-dihydric phenols are inhibitory, then the relative concentration of these substances could determine the effectiveness of the bypass. In this respect, it is interesting that root extract, but not seed extract, accelerated the system. Furthermore, Raoul (26) found that the hordenine content of barley roots varied with age, being nil in seeds, reaching a maximum between 4 and 7 days, and then slowly declining to reach zero after about 30 days. This kind of change in concentration of substances having cofactor activity could be the basis of reported changing patterns of terminal oxidation during root development.

Summary

Homogenates of roots of barley seedlings contain an enzyme system which catalyzes the rapid oxidation of reduced pyridine nucleotides. Most of this activity is recovered in the supernatant fraction after high-speed centrifugation. Crude preparations of the oxidase require no added cofactors to catalyze the oxidation of DPNH to DPN^+ with the uptake of 0.5 mole of O_2 per mole, but the rate of the reaction is greatly increased by the addition of a boiling water extract of barley roots. Such extracts are also effective in restoring activity to enzyme preparations which have been inactivated by dialysis. These effects of root extract could not be duplicated by extracts of barley seed, extracts of barley root ash, or by any of several cofactor preparations.

Hordenine and tyramine, phenolic alkaloidal amines found in barley roots, stimulate both the rate of DPNH disappearance and that of O_2 uptake, but cause no stoichiometric changes. These compounds, therefore, may be naturally occurring cofactors of the oxidase system. A requirement for additional heat-stable, dialyzable cofactors is indicated by the inability of the alkaloidal amines to reactivate dialyzed enzyme preparations unless root extract also is present.

The DPNH oxidase system is inhibited by cyanide, ascorbic acid, hydroquinone, diethyldithiocarbamate, and by various *ortho*-dihydric phenols; it is accelerated by hordenine, tyramine, and some other monohydric phenols. Non-phenolic derivatives of the inhibitory or acceleratory phenols are relatively innocuous. This behavior suggests that monohydric phenols may function as carriers of electrons in the system.

The terminal enzyme of this DPNH oxidase system has not been identified, but the present evidence does not support any of the terminal oxidases which are frequently suggested as participants in plant respiration.

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GROWTH REGULATORS AND FLOWERING. II. THE COBALTOUS ION [1, 2]

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It was shown previously that cobaltous ion inhibits flowering of the short-day plant cocklebur if it is applied to plants at concentrations of about 3×10^{-4} to 6×10^{-2} moles per liter just before a single inductive dark period (10). Although development of the floral bud is inhibited by extremely high concentrations of cobaltous ion applied at any time, lower concentrations are only effective in floral inhibition providing they are applied before the end of the critical night (the minimum period of darkness required to produce flowers). Cobaltous ion increases the duration of the critical night—that is, the biological timing mechanism in the leaf of the cocklebur appears to be slowed down by cobaltous ion (fig. 1, discussed below, illustrates this phenomenon).

The literature contains more than 60 references relating to the effect of the cobaltous ion upon plants. Most of these are concerned with nutrition, although some do attempt to ascertain the mechanism of action in the response under consideration. Some five mechanisms have been suggested, and the first four of these were investigated in the present study, in regards to the action of cobaltous ion in the flowering process:

1) An inhibition of peroxigenesis. Galston and Siegel (3) reported that cobaltous ion inhibited the formation of organic peroxides in pea stem sections and thus spared the destruction of indole acetic acid by indole acetic acid oxidase. They further reported that manganous ion would act in an opposite or even antagonistic manner in this tissue.

2) Oxidation of phenol type compounds. Gillette et al (4) reported that Mn^{++}, Co^{++}, Ni^{++}, Ca^{++} and Fe^{++} catalyze the oxidation of certain phenolic type compounds. They are effective in the order shown.

3) A promotion of metabolism. Thimann (12) demonstrated that the promotion by cobaltous ion of elongation of *Avena coleoptile* sections or etiolated pea sections could be reversed by dinitrophenol. He concluded that cobaltous ion might channel respiratory energy into growth.

4) Relation of cobaltous ion to endogenous sulfhydryl compounds. Nickerson and van Rij (8) reported that cobaltous ions inhibit cell division and increase the production of mycelial forms in yeast. They were able to reverse the effects of cobaltous ion with cysteine. They concluded that cobaltous ion acts through an influence on sulfhydryl groups, which are in some way important in cell division. Thimann (12) reported that concentrations of cobaltous ion high enough to be inhibitory to the growth of etiolated pea sections probably act through an effect on sulfhydryl compounds, since he was able to reverse this effect with 2,3-dimercaptopropanol (BAL).

5) Inhibition of synthesis and regeneration of cytoplasmic proteins. Werz (14) reported that application of cobaltous ion to a marine green alga immediately stopped regeneration and synthesis of cytoplasmic proteins. Although such an effect was not tested in the present system, cytochemical responses should perhaps be kept in mind in future interpretation of cobaltous ion effects.

Since knowledge of the timing mechanisms of photoperiodism has increased through experiments utilizing light interruption of an inductive dark period, experiments reporting the effects of cobaltous ion in these responses are discussed in this paper. The timing mechanisms which control endogenous daily leaf movements are considered by some authors (1) to be related to timing in flowering. Thus effects of cobaltous ion upon daily leaf movements were also studied.

It will be shown that the mechanism of action of cobaltous ion in its disturbance of timing in the photoperiodic response of cocklebur is probably related to sulfhydryl groups in the leaf cells, that timing is not reversed by brief light interruptions of the inductive dark period, and that timing in the flowering process can be separated from timing of daily leaf movements.

It should be noted that cobaltous ion, since it is not an organic substance, is eliminated by the report of the special committee on definitions (Plant Physiol. 29: 307. 1954) from the class of compounds known as growth regulators. Yet its regulatory effects upon flowering are similar in many respects to effects of the usual growth regulators. Thus it is convenient to discuss these effects in this series.

MATERIALS AND METHODS

The general procedure followed in most of the experiments has been described in detail previously (9, 10, 11). In all experiments, cocklebur plants (*Xanthium pennsylvanicum* Wall.) were grown from

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seed and maintained in a vegetative condition by supplementary light (total day length of 17 to 21 hours). A "pinch" of commercial fertilizer (Vigoro) mixed with about an equal amount of NH_4NO_3 or NH_4SO_4 was placed in each pot at least a week before an experiment. Plants were prepared for an experiment by sorting them according to the size of the half-expanded leaf and then removing all leaves below this leaf and the one above it. In dark weather, a better response can be obtained by preparing plants 1 day before the single extended dark period, used to induce plants to flower. Chemicals were applied just before induction by dipping the leaf for 1 or 2 seconds in a solution of the chemical containing 6 drops of detergent per liter (Tween-20). Nine days following induction, apical buds were examined under a dissecting microscope and classified according to a previously described (9) system of stages of development of the apical meristem.

Light interruptions during the dark period (figs 5, 6, and 7) were carried out with four 300-watt reflector flood lamps enclosed above a layer of water approximately 3 centimeters thick and surrounded by cooling coils arranged so that water condensing on them dripped back into the layer below (15). Duration of light interruption is controlled by an interval timer. An even distribution of red light is obtained by using a piece of translucent red plastic. Far-red light is obtained by adding to the above system a double layer of blue cellophane sandwiched between

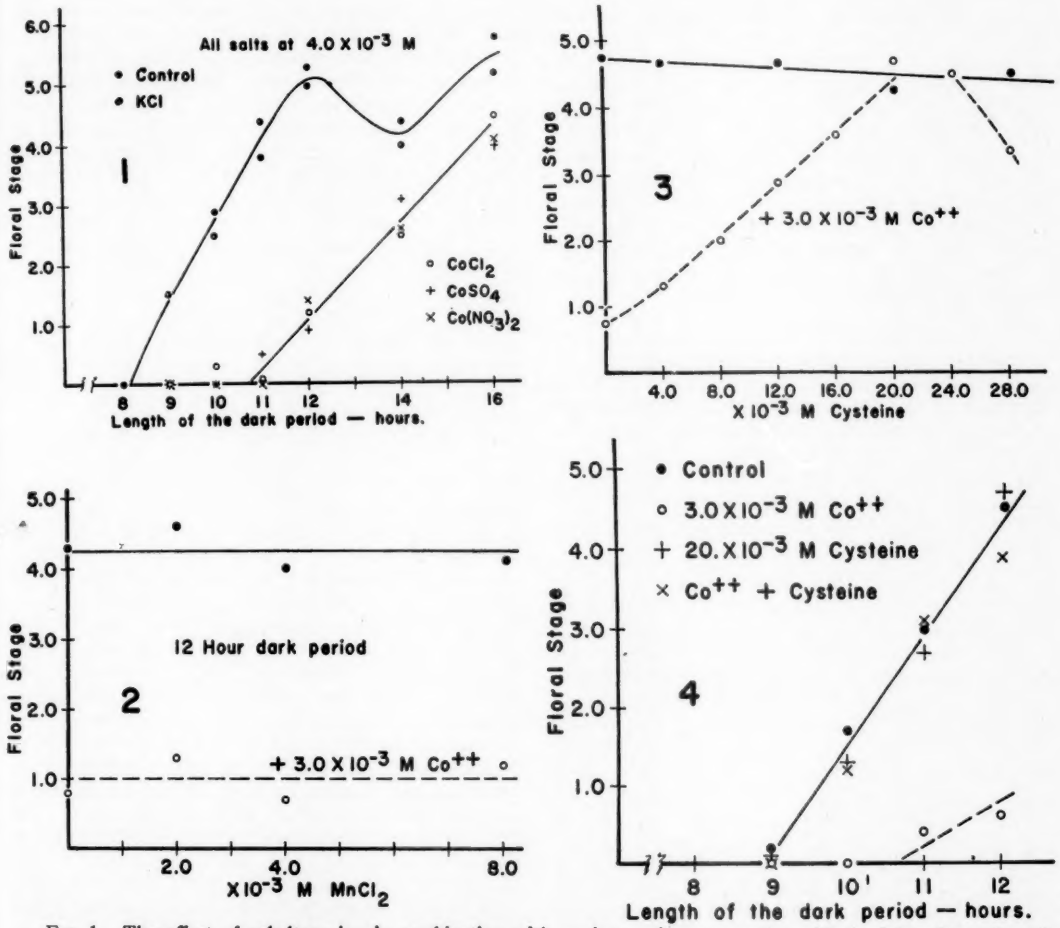


FIG. 1. The effect of cobaltous ion in combination with various anions upon the critical night. Data for all experiments are given in table I.

FIG. 2. The effects of manganous ion, and manganous ion plus cobaltous ion upon flowering of cocklebur.

FIG. 3. Reversal of cobaltous ion inhibition of flowering in cocklebur by cysteine.

FIG. 4. The effects of cobaltous ion, cysteine, and cobaltous ion plus cysteine upon the critical night.

2 thin layers of clear plexiglass. The red plastic begins to transmit at 5800 Å and transmits more than 90 % of the light with wave lengths above 6800 Å (thus "red" in this paper actually means red plus far-red). The combination of red plastic plus blue cellophane transmits less than 10 % below 6800 Å and more than 80 % above 7500 Å.

In the leaf movement experiments (fig 8), the position of the leaf is observed with the plants placed in front of a light background and behind a sheet of glass covered by a piece of Saran Wrap (a transparent film produced by the Dow Chemical Company) which is attached by its own adhesion to the glass surface. The outline of stem, petiole and blade is then traced on the Saran Wrap with a red wax pencil. Weak green light is used for observations during the dark period. The time and temperature are recorded, and the number assigned to each plant is written beside its outline. The best angle to measure proved to be one between the stem above the axil and an imaginary line from the axil to the tip of the leaf. Five different observers, each of whom performed all operations described above, produced average values for 10 plants which agreed within 5 degrees. The measured angle varies about 60° (at least 10 times the margin of error) in each 24 hour period, although the actual position of the leaf varies even more (from an almost horizontal position in the afternoon to the near vertical some time after midnight).

Details such as leaf size, time of year, etc. are shown for all experiments in table I.

Experiments and Results

Effect of Various Salts of Cobalt upon the Critical Night: In previous experiments by the author (10), cobaltous ion has always been applied as the chloride. To demonstrate that the action was due to cobaltous ion rather than the chloride ion, plants were treated with a number of salts of cobalt just before dark periods of various lengths. Control plants received no treatment or were treated with potassium chloride. As is evident in figure 1, the 3 salts containing cobalt all extended the critical night approximately 3 hours compared to the control or to the potassium chloride treatments.

Table I

Data Relating to Experimental Conditions for Results Shown in Figures 1 to 8

Figure number	Expt. number	Date *	Plants per treatment	Leaf left on plants **	Date plants trimmed
1.	C–115	7/23/58	10	T–#3	7/22/58
2.	C–119	8/2/58	10	T–#3	8/1/58
3.	C–123	10/8/58	20	T–#3	10/7/58
4.	C–125	10/17/58	10	T–#3	10/16/58
5.	C–64	8/8/57	16	T–#3	8/8/57
6–A.	C–69	10/3/57	12	S–#3	10/2/57
6–B.	C–57	7/11/57	8	T–#3	7/11/57
7.	C–84	12/21/57	10	T–#3	12/20/57
8.	C–111	4/15/58	5	S–#3	4/12/58

* Date plants were placed in cabinets.

** Numbers refer to length of leaf midrib of the most rapidly expanding leaf as follows: S–#3: 5.9 to 7.7 cm.; T–#3: 6.9 to 8.5 cm.

Mechanism of Action of the Cobaltous Ion: The first 4 proposed mechanisms of action of cobaltous ion outlined in the introduction were tested as follows:

1) *Inhibition of peroxigenesis*: Since Galston and Siegel (3) reported that manganous ion might act in an opposite or even antagonistic manner to cobaltous ion in pea stems, a series of plants was treated with a solution of cobaltous chloride alone, in combination with various concentrations of manganous chloride, or with various concentrations of manganous chloride alone. A 12-hour dark period (a convenient night length for demonstration of the cobaltous ion inhibition—fig 1) was used. The results are shown in figure 2. It is obvious that manganous ion neither influences flowering, nor does it overcome the inhibitory effect of cobaltous ions.

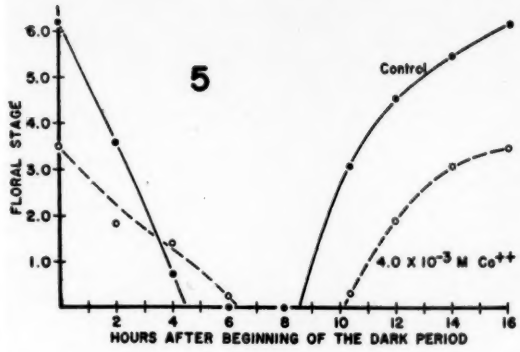
2) *Oxidation of phenol type compounds*: Manganous ion was most effective in the experiments of Gillette et al (4), yet it has no effect on flowering. Thus the experiment of figure 2 seems to eliminate this mechanism of action from consideration.

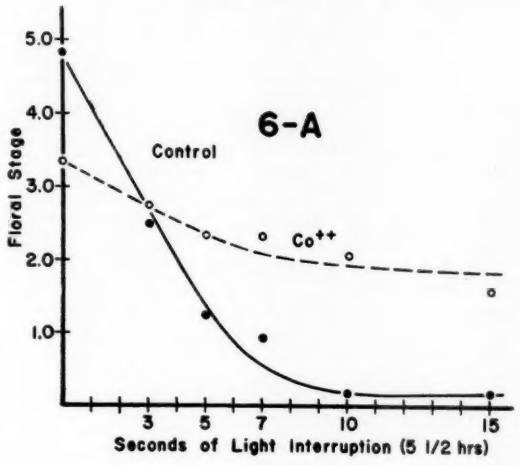
3) *A promotion of metabolism*: Thimann's (12) conclusion that cobaltous ion might channel respiratory energy into growth was based upon a reversal of the cobaltous ion effect by 2,4-dinitrophenol (DNP). Thus an experiment was set up in which the critical night was determined (as in fig 1) for a set of control plants, for plants treated with DNP, for plants treated with cobaltous ion, and for plants treated with cobaltous ion plus DNP. No reversal of the cobalt effect by DNP could be observed. It would indeed be very surprising if such reversal could be observed, since dinitrophenol is itself inhibitory to flowering (10).

4) *Relation of cobaltous ion to endogenous sulfhydryl compounds*: The possibility that this relationship might be important in the cobaltous effect upon flowering was tested by treating plants with cobaltous ion, cysteine (8), and both substances applied in the same solution, as in the manganous ion experiment described above. From the results shown in figure 3, it is evident that cysteine is able to completely reverse the effects of cobaltous ion upon flowering. Glutathione has been used with equal success. It is important to note that the vegetative responses to cobaltous ion (slight yellowing of leaf edges) were not reversed by cysteine or by glutathione. Thus cysteine must not influence penetration of cobaltous ion or its effect upon leaf cells.

Figure 4 shows the effects of cobaltous ion, cysteine, and the combination upon the critical night. Again it can be seen that cysteine completely reverses the effects of cobaltous ion upon flowering.

Interaction of Cobaltous ion and Light: Experiments designed to study the interaction of





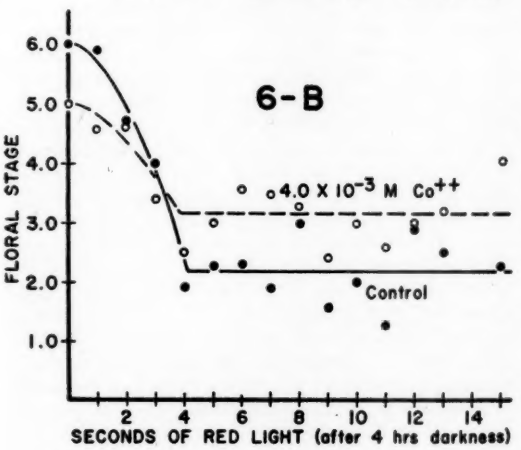
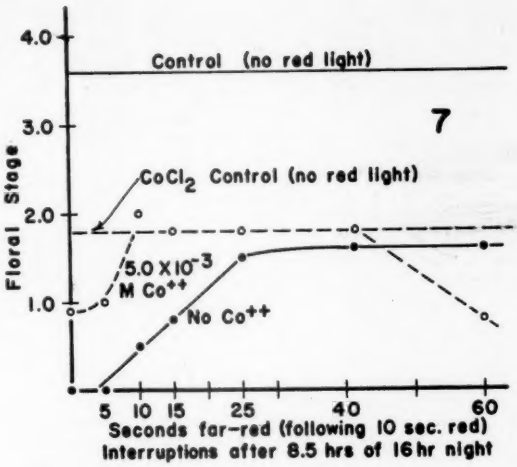


FIG. 5. The effects of a 10-second red light interruption given at various times during a 16-hour inductive dark period upon the subsequent flowering of plants treated or not treated with cobaltous ion. Red light source is described in the text.


Control (no red light)
7
CoCl₂ Control (no red light)
5.0 X 10⁻³ M Co⁺⁺
No Co⁺⁺
Floral Stage
Seconds far-red (following 10 sec. red) interruptions after 8.5 hrs of 16 hr night

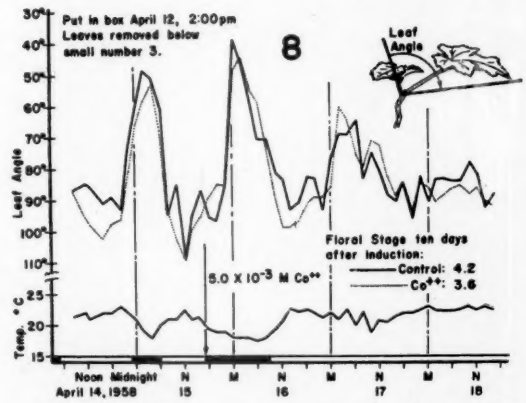


FIG. 6. A. Effects of various durations of a red light interruption given 5.5 hours after the beginning of a 16-hour inductive dark period upon flowering of control plants and plants treated with 5.0×10^{-3} M cobaltous ion. The points representing response of cobaltous ion treated plants do not indicate the exact duration of light required for saturation. They do, however, show that cobaltous ion treated plants are less sensitive to red light interruption at this time than nontreated plants.

FIG. 6. B. The same type of experiment as figure 6-A except for the variables as noted. Horizontal lines represent averages of all points from 4 seconds to 15 seconds. In spite of the scatter in these points, the protective action of cobaltous ion is evident and the saturating durations appear to be the same for both treated and control plants.

FIG. 7. The reversal of red light inhibition with far-red light for control and cobaltous ion treated plants.

FIG. 8. Leaf movements in cocklebur as influenced by an inductive dark period and cobaltous ion. Dark periods are indicated by black areas on the abscissa. Light periods (light areas) consisted of approximately 500-ft-c of white light illumination from four 300-watt reflector flood lamps installed as described in the text.

cobaltous ion and light are quite difficult due to introduction of 2 more factors into a system already containing a number of uncontrolled variables (e.g. natural light intensity). Thus it has been necessary to repeat each experiment many times. Some of the results which are common to most of the experiments performed so far are of interest here.

Light interruption at various times during the dark period: At various times during a 16-hour dark period, groups of plants treated with cobaltous ion and control plants were illuminated with red light for a brief interval (8 to 15 seconds in different experiments). This experiment has been repeated 6 times. Representative results are shown in figure 5. Light interruptions during the last half of the dark period produce an effect upon flowering very much like that shown in figure 1; i.e. cobaltous ion increases the critical night. If light interruption is given during the 1st half of the dark period, however, the inhibitory effects of light and cobalt are not additive. Indeed, during a short period, plants treated with cobaltous ion are less sensitive to light than untreated plants. This effect has been observed in all experiments designed to test it.

Light interruptions of varying duration: At an appropriate time during the 1st half of a 16-hour dark period, light interruptions of various duration were given to treated and nontreated plants. Since light interruption was given on the steep part of the curve shown in figure 5 (plants interrupted at 8 hours usually are completely vegetative, and the saturating amount of light cannot be determined), the timing requirements were particularly strict. This experiment has been repeated 5 times and the results of 2 such trials are shown in figures 6-A and 6-B.

The control curves show the typical response: increasing duration of light interruption results in increased inhibition up to a duration of about 4 seconds (fig 6–B) to 8 seconds (fig 6–A). After this time, increased duration of light interruption does not increase the inhibition of flowering. It should be noted that saturating durations of light are less effective in floral inhibition for plants treated with cobaltous ion than for untreated plants. This effect can be observed in all experiments, providing light interruption is given at the appropriate time during the dark period.

Cobaltous ion and far-red reversal of red inhibition: It has been shown by Downs (2) that the inhibition of flowering produced by red light interruption of the dark period may be reversed, to some extent at least, by far-red illumination following this interruption. The effect of cobaltous ion upon this response was studied. Cobaltous ion treated and control plants were illuminated first with a constant (usually 10 seconds) interruption by red light near the middle of the 16-hour dark period. Immediately following the red light interruption, far-red light was applied for periods of varying duration. This experiment has been repeated 5 times.

Although no two of these experiments have agreed in all particulars, two features common to most of them are illustrated by figure 7. First, the reversal of red light inhibition of control plants by far-red light is never complete (see Downs, (2)). In some experiments, reversal may be nearly 80 % of the control, but the reversal shown in figure 7 is quite typical. Second, the reversal of cobaltous ion treated plants to the flowering level of the cobaltous ion controls (no interruption), is often complete or nearly so. A third effect, shown by figure 7, which did not appear in all experiments of this type was a shorter saturating duration of far-red light required for reversal of cobaltous treated plants than for reversal of control plants.

Cobaltous Ion and Leaf Movements: Leaf movements were observed for 2 sets of plants. After observations had shown that leaf movements of both sets were in reasonable synchronization, 1 set was treated with cobaltous ion and both sets were given a single 16-hour inductive dark period. Leaf movements and temperatures before, during and following this dark period are shown in figure 8, and the floral stages are also recorded in this figure. It can be seen that cobaltous ion inhibited flowering to the degree to be expected with a 16 hour night and the concentration of cobaltous ions which were used. Leaf movements, however, were not significantly influenced by treatment with cobaltous ion. This experiment has been repeated 3 times.

Discussion and Conclusions

Although each of the experiments illustrated by figures 1, 5, 6 and 7 illustrates a different aspect of the problem, all demonstrate the phenomenon that cobaltous ion slows down the time measuring reactions of floral induction. Figure 5 most clearly illustrates the kinetics of this phenomenon. What is the significance of these experiments for our understanding of the flowering process?

Previous experiments by the author on light interruption given at various times during the inductive dark period have yielded curves which are almost symmetrical with respect to the 1st and 2nd halves of the dark period (11). These experiments were interpreted on the basis that the effectiveness of a light interruption applied during the first part of the dark period depended upon the amount of darkness following this light interruption. Thus plants interrupted 4 hours after the beginning of a 16-hour dark period, plants receiving a total of 12 hours of darkness with no light interruption, or plants which were interrupted after 12 hours of a 16-hour dark period all flowered about the same. It was assumed that light interruption during the first part of the dark period reversed the reactions of the dark period, so that they would have to begin anew following the light interruption (10). This explanation seemed adequate, although in some experiments curves were not quite symmetrical.

The experiments of which figure 5 is typical cannot be interpreted on this basis. Light interruption

of cobaltous ion treated plants at various times during an inductive dark period yields curves which are very markedly asymmetrical. Indeed, in some experiments (not shown) plants treated with cobaltous ion flower to some degree even when they are interrupted 8 hours after the beginning of a 16-hour dark period, although control plants never flower under these conditions. Plants treated with cobaltous ion also never flower if they are given only 8 hours of darkness.

Figure 5 indicates that the effectiveness of a light interruption applied before the completion of the critical dark period is not dependent upon the duration of the dark period following this interruption, but upon the timing mechanisms of flowering and, of course, upon the total amount of darkness received by the plants. It appears, then, that light interruption during the first part of the dark period may not reverse all of the "dark reactions" in the flowering process. The "clock" may not be reset. It would appear from the experiments of Hamner (5) and those of Liverman and Bonner (6) that high intensity light, or an energy substrate such as sucrose, will reset the clock, but the data of figure 5 indicate that a light interruption of short duration probably does not.

It has been shown previously that the timing mechanism of the flowering process must not be the time required for the photoreceptor pigment to be converted in the dark from a far-red to a red receptive form (11). Thus pigment conversion appears to be complete in something less than 3 hours rather than the 8.5 hour period measured by the timing mechanism of flowering.

In figures 6 and 7 it is possible to estimate the saturation times for red interruption of the inductive dark period or for far-red reversal of red inhibition. These saturation times are an indication of the total energy required to saturate the pigment system and are, in a sense, a measure of the amount of photoreceptor pigment present. Most experiments performed so far indicate that cobaltous ions do not influence these saturation times, although the data remain inconclusive. At any rate, the kinetics of light interruption with red light or with red light followed by far-red light do not seem to be changed substantially by the presence of cobaltous ions.

It is tempting to envisage mechanisms involved in the light interruption phenomena. Schemes come to mind to explain how light interruption might act to inhibit flowering in some manner other than by "resetting the clock." Red light, by converting the photoreceptor pigment to the far-red receptive form, might cause a synthesis of some substance inhibitory to flowering or to subsequent synthesis of flowering hormone. The amount of inhibitory substance produced would have to be a function of the timing reactions of flowering which are sensitive to cobaltous ion. Inhibiting substances have often been postulated as part of the flowering mechanism (e.g. (13)).

James Bonner (personal communication) has suggested a similar scheme in which the timing mechanism is not reset by red light interruption. The timing mechanism controls the amount of flowering hormone synthesized, beginning at the end of the critical night and continuing thereafter at a decreased rate. A red light interruption "instructs" the leaf not to synthesize flowering hormone for some time interval after the light interruption (e.g. a substance may be produced by the light flash which inhibits flowering hormone synthesis and requires a time interval to be removed from the leaf). At the end of the time interval, rate of flowering hormone synthesis depends on the length of time since the beginning of the inductive dark period—as measured by the "biological clock." Cobaltous ion slows down the "biological clock" and possibly increases the time interval required until flowering hormone synthesis may begin following a light flash. Such a scheme, worked out graphically, elegantly explains the asymmetry of the curves in figure 5.

It is also possible to devise schemes in which the clock is reset, but the assumptions seem even more complex than those discussed above.

It is difficult to see how such schemes might be tested, except that the data upon which they are based might be improved by better environmental control. Furthermore, figures 3 and 4 indicate that the observed responses are in some way related to endogenous sulfhydryl groups. A biochemical approach to the problem might give us further insight into the nature of cobalt-influenced light responses such as timing in the flowering process and leaf expansion.

Figure 8 indicates that the timing reactions of flowering may be separated from the timing reactions which control leaf movements. Cobaltous ion was ineffective in disturbing the timing of leaf movements, although timing in the flowering process is clearly upset. Dr. Franz Leinweber reports (personal communication), however, that he has been able to slow down leaf movements in the cocklebur by application of cobaltous ion. His experiments were carried out at constant temperature with accurately controlled environmental conditions which were quite superior to those used by the present author. It may be that the timing mechanisms controlling leaf motions in figure 8 are influenced by the sharp variations in temperature as shown in that figure. Thus it may be necessary to manipulate temperature as an experimental variable in order to separate effects of cobaltous ion upon timing of leaf movements from effects upon timing in flowering. Nonetheless it may be significant that such effects can be separated at all.

Summary

1. Cobaltous ion extends the critical night, the minimum single period of darkness required by the cocklebur for manifestation of the first perceptible signs of flowering. The critical night is extended by cobaltous ion in the presence of a variety of anions, and potassium or manganous ions have no effect upon the critical night.

2. Biochemical mechanisms of action of the co-

baltous ion in the flowering process were investigated. Since manganous ion has no effect upon flowering either in the presence or absence of cobaltous ion, it was concluded that cobaltous ion does not exercise its effects upon the flowering process through the indoleacetic acid oxidase system, nor by oxidation of phenol type compounds. Dinitrophenol does not reverse the effects of cobaltous ion in flowering, and thus these effects are probably not concerned with the promotion of metabolic activity. The action of cobaltous ion in the flowering process can be completely reversed by cysteine and glutathione (8). Thus it is concluded that the mechanism of action of cobaltous ion in the timing reactions of flowering is some way related to endogenous sulfhydryl groups.

3. The effects of cobaltous ion upon the timing reactions of the flowering process were studied by kinetic experiments on the effective times of red light interruption of the inductive dark period, the effect of duration of such an interruption given at a specific time, and the reversal of red interruption with far-red. The effect of a low intensity, red light interruption of the inductive dark period depended not only upon the total length of this dark period, but upon the extent to which the timing reactions had been completed at the time of the interruption. Thus the effectiveness of the interruption cannot be understood only by taking into account the length of the period of darkness following or preceding this interruption. It seems likely that such an interruption does not completely reverse the timing reactions which are begun at the time the plant was placed in darkness.

4. Although the timing mechanisms of flowering are strongly influenced by the cobaltous ion, the timing mechanisms of leaf movements were not so affected in experiments carried out under varying temperature conditions. It may be significant that effects upon flowering and leaf movements can be so separated.

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FLOWERING IN FRUIT TREES AND ANNUAL PLANTS AS AFFECTED BY PURINES, PYRIMIDINES AND TRIIODOBENZOIC ACID[1]

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In previous papers it was reported that uracil (10) and caffeine (14, 15) enhance the syntheses of both ribonucleic acid (RNA) and protein in various fruit tree species. Similar results were obtained with xanthine, while adenine and guanine, when applied at a rather early developmental stage of the plant, promoted the synthesis of deoxyribonucleic acid (DNA) (11). In addition it was later found by us that fruitful and non-fruitful trees differed in their relative levels of uracil (in some species fruitful species contained up to 80 to 170 % more uracil than their non-fruitful counterparts), and, in the case of olive trees, also in their xanthine levels. As a result of the above findings xanthine and uracil were applied to plants and were found, in some cases, to enhance flowering.

These latter preliminary experiments were now extended to more species of deciduous and evergreen fruit trees as well as to a series of annual plants. In part of these experiments 2,3,5-triiodobenzoic acid (TIBA) which was found to promote flowering in grape vines (1) and several other plant species (4, 9) was also used for comparative purposes.

MATERIALS AND METHODS

For these experiments the following fruit tree species were chosen: 13-year-old olive trees var. Sevillano were treated in January 1955 and flowered in April 1955; Olive trees var. Souri were treated in September 1955 and flowered in April 1956; 12-year-old grape vines var. Dabouki (in this variety the basal buds of the branches are in general non-fruitful) were treated in May 1957 and flowered in April 1958. Similar experiments were conducted with 1-year-old grapefruit seedlings which were treated at various times from September 1956 until February 1957. As annual plants, potato and bean plants were chosen and were treated in May 1957. All these plants were sprayed with 50 ppm aqueous solutions of uracil and xanthine, respectively. In addition, other plots were sprayed with 50 ppm solutions of adenine, guanine, caffeine and uric acid. The experiments with olive trees were carried out in 4 replicates, each replicate being comprised from 5 one-year-old 30 cm long shoot sections, i.e. each treatment was thus applied to a total number of 20 shoot sections. The shoots were selected at random around the trees at 1.50 meter height above the soil, and the treatments and replicates were distributed at random among them. Flowering is expressed as number of flowers per replicates.

The experiments with grape vines were carried out in 20 replicates, each replicate being comprised from 1 vigorously growing branch; i.e. each treatment was applied to a total of 20 branches, and in table 2 average figures of the 20 replicates of each treatment are represented. With grapefruit seedlings 8 replicates were used and with potatoes 6 replicates, each replicate being composed of 3 plants. With the bean plants 2 successive experiments were carried out, each one with 25 plants for each treatment. Both experiments gave similar trends and the results of 1 experiment are represented. All treatments and replicates were completely randomized. The number of flower clusters was counted at the time of the natural flowering season.

One month after treatment leaf samples were collected from the olive trees and grape vines and analyzed for RNA, (10) DNA (11), and alcohol-insoluble nitrogen (10). From the olive trees leaves for the analyses were collected at random from the treated shoot sections. In case of grape vines only leaves from the 1st basal buds were harvested. For the analysis of the nucleic acids the leaf samples were dropped into boiling ethanol for 2 minutes immediately after their detachment from the plants. Then the tissue was extracted 2 times with ice cold 0.2 *N* perchloric acid and 3 times with an ethanol : ether (3:1) mixture at room temperature. The extraction of the nucleic acids from the defatted tissue of the evergreen olive trees was slightly different from that of grape vines. The extraction of nucleic acids from the evergreens was accomplished with 1 *N* perchloric acid for 0.5 hour at 70° C. This procedure was repeated 3 times. For the other plants it was found necessary to lower the temperature; again 3 extractions were made, but in this case for 1 hour at 57° C. The supernatants from all 3 extractions were combined and the nucleic acids were then fractionated under mild basic conditions for 15 hours at 37° C, after which DNA was precipitated with perchloric acid at pH 2. In the supernatant, RNA was estimated with the orcinal reagent (16). The precipitated DNA was redissolved at pH 6 and determined with the indole reagent (5).

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Results and Discussion

The first experiments were carried out with olive trees which were treated with adenine, guanine, xanthine, uracil, caffeine and uric acid. It can be seen from table I that, in the olive trees, both xanthine and uracil were effective in promoting flowering, but that the response to xanthine was stronger. Potato and bean plants (table II) also responded to xanthine and uracil; uracil was significantly more effective with potatoes but bean plants responded quite well to both uracil and xanthine. In the grape vine (table III) caffeine and uracil promoted flowering. Olives also responded to caffeine, while the effect of uric acid was insignificant.

Another set of experiments was carried out with young trees. For this purpose we selected grapefruit seedlings. It is occasionally found that grapefruit seedlings produce flowers when only a few months old (3, 7, 17), although this phenomenon is not repeated and the trees do not flower again until maturity (17). Grapefruit seeds were sown in March. A part of these seedlings flowered during June (15 % of the seedlings, a percentage similar to what was observed by Van der Meulen-17). After transplantation these seedlings were sprayed at various times, from November 1956 until February 1957, with 50 ppm solutions of xanthine and uracil. Part of these seedlings also were treated with 50 and 500 ppm solutions of TIBA. Table IV gives the number of flowering plants as a percentage of the total number of seedlings of the respective treatments, combining all spraying periods. In conformity with the results summarized in tables I, II and III it is seen from table IV that both uracil and xanthine promoted flowering in the young seedlings, but in this case xanthine was more effective. It must be pointed out that the greatest percentage of flowering seedlings, amounting up to 50 % of the total flowering seedlings of the xanthine treatment, were obtained by the November spray. Spraying the same seedlings again later in the summer as well as spraying 4-year-old grapefruit trees with up to 150 ppm xanthine, did not result in any flowering. A still greater effect of xanthine was observed when the number of flowers per seedling was taken into account.

In the case of grapefruit seedlings the flowering response to xanthine and uracil was compared to that of TIBA. From table IV it is seen that while 50 ppm of TIBA was quite uneffective, the higher concentration (500 ppm) gave about the same effect as xanthine. Different concentrations of TIBA if combined with uracil and xanthine were uneffective except one combination (500 ppm TIBA + 50 ppm xanthine) to which the grapefruit seedlings responded about as much as to xanthine and TIBA alone.

It is of interest to point out that both evergreens, olives and grapefruits, were more responsive to xanthine, in contrast to the grape vines and the an-

Table II

Effect of Purines and Pyrimidines upon the Flowering of Potato and Bean Plants

Treatment	Bean	Potato
Control	4	11
Adenine	5	16
Guanine	...	0**
Xanthine	8	27**
Uracil	9**	50**
Caffeine	6	30**

* Expressed as no. of flowers per plant.

** Significantly different from control at 5 % level.

Table I

Effect of Purine and Pyrimidine Bases upon Flowering, RNA/DNA Ratio and Protein-N of Two Olive Varieties

Treatment	No. of flowers	RNA (mg/g dry matter)	DNA (mg/g dry matter)	RNA / DNA	Protein N (% of dry matter)	Protein N (% of total N)
Var. Souri						
Control	42	5.3	1.0	5.6	1.89	73
Adenine	38	5.6	1.1	4.9	1.98	69
Guanine	32	4.0	1.0	4.1	1.72	71
Xanthine	117	6.8	0.9	7.9	1.63	88
Uracil	105	6.4	0.9	7.3	1.69	85
Caffeine	82	5.4	0.8	6.5	1.55	81
Uric Acid	48	5.3	1.0	5.3	1.70	70
L.S.D. *	43	0.1	0.1	...	0.11	...
Var. Sevillano						
Control	28	3.7	0.8	4.6	2.11	70
Adenine	20	4.3	0.9	4.7	2.20	72
Guanine	33	4.7	0.8	5.6	1.95	75
Xanthine	74	6.9	0.8	8.9	2.08	91
Uracil	62	6.2	0.8	8.1	1.98	83
Caffeine	84	5.6	0.8	7.5	1.95	86
L.S.D. *	28	0.2	0.1	...	0.17	...

* Least significant difference.

TABLE III

EFFECT OF PURINE AND PYRIMIDINE BASES UPON FLOWERING AND THE RNA/DNA RATIO OF GRAPE VINES

TREATMENT	NO. OF GRAPE BUNCHES (PER BRANCH)	RNA (MG/G DRY MATTER)	DNA (MG/G DRY MATTER)	RNA/DNA	BEARING BRANCHES*
Control	1.30	7.1	1.1	6.3	28
Adenine	1.41	6.9	1.0	6.8	25
Guanine	1.29	7.6	1.2	6.2	19
Xanthine	1.57	7.2	1.0	7.2	22
Uracil	2.85	9.3	1.1	8.6	54**
Caffeine	2.17	6.9	1.0	7.0	39**
L.S.D.	0.73	0.1	0.033	...	

* As % of total branches of first node.

** Significantly different from control at 5 % level.

nual plants where uracil was found to be more effective.

There seems to exist a similar trend in the effects of uracil and caffeine upon flowering and its promoting influence on the syntheses of RNA and protein (10, 14, 15). Flowering usually starts as the plant matures, after a period of vegetative development (4, 6). With regard to the latter point it was found that in the course of the natural maturation, as well as under the influence of caffeine (14, 15), the RNA/DNA ratio increases. In the present work a similar effect was found for uracil, xanthine and caffeine (tables I and III), suggesting certain relationships between maturation, flowering and RNA. The existance of some relationships between uracil and flowering is also suggested by a recent report (18) where an uracil derivative, 5-fluorouracil, was found to inhibit floral induction while a certain reversal of this inhibition was obtained with uracil.

It has repeatedly been outlined for various other organisms (18, 19) as well as for plants (20, 21) and fruit trees (10, 12, 13) that RNA apparently is intimately involved with protein synthesis. A certain parallelism between RNA/DNA and protein has also been found in this work (tables I and III) and it might well be that the effect of the flowering-promoting purines and pyrimidines comes about through their enhancing effect upon the synthesis of RNA and protein, and through the latter, in some way, upon the synthesis of the flowering hormone which apparently takes place in the leaf.

Investigations are at present in progress to study the relationships between the 3 variables, RNA, protein and flowering, as well as the part played by the chemical configuration of the effective substances in evergreen and deciduous plants.

TABLE IV

EFFECT OF XANTHINE, URACIL AND TRIIODOBENZOIC ACID UPON THE FLOWERING OF 1-YEAR-OLD GRAPEFRUIT SEEDLINGS

TREATMENT	NO. OF FLOWERING PLANTS *
Control	5.5
Xanthine 50 ppm	20.0
Uracil, 50 ppm	13.3
TIBA, 50 ppm	10.0
TIBA, 500 ppm	22.0
TIBA, 50 ppm + Xanthine, 50 ppm	0
TIBA, 500 ppm + Xanthine, 50 ppm	22.2
TIBA, 50 ppm + Uracil, 50 ppm	5.8
TIBA, 500 ppm + Uracil, 50 ppm	5.8

* As % of total no. of plants of each treatment.

SUMMARY

The effects of various purines and pyrimidines upon the flowering of several fruit tree species and annual plants were tested. Uracil, xanthine and caffeine were found to be active while adenine and guanine had no effect on flowering. In the evergreen, olive and grapefruit trees, xanthine was more effective than uracil while in grape vines and in the annual potato plants uracil was more promotive. The increase in flowering as brought about by xanthine, uracil and caffeine was paralleled by an increased protein-N and RNA/DNA ratio. A possible relation of flowering to the increased RNA/DNA ratio has been suggested.

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MOLYBDENUM-MANGANESE-IRON ANTAGONISMS IN THE NUTRITION OF TOMATO PLANTS [1]

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Antagonisms and interactions among the essential elements molybdenum, manganese, and iron and their effects on plant growth have been reported by a number of investigators. Millikan (11), for example, with solution culture experiments concluded that molybdenum would alleviate iron deficiency symptoms in flax induced by excess quantities of manganese, zinc, copper, nickel, or cobalt. Relatively high concentrations of molybdenum (5 to 25 ppm added to the nutrient solution) were required. In a subsequent publication, Millikan (12) reported a similar molybdenum-manganese interaction in solution culture experiments with peas, cabbage, and tomatoes, and that in both field and pot experiments manganese toxicity to flax growing in highly acid soils was alleviated by additions of molybdenum.

Other investigators Hewitt (5, 7), Mulder (13) and Warrington (16) obtained results in direct contrast to those by Millikan; molybdenum, rather than alleviating iron chlorosis induced by high concentrations of metals, accentuated the chlorosis and led to further decreases in growth. Later Warrington (17) reported results to some extent contradictory to those obtained earlier and in agreement with Millikan's proposals. In studies of molybdenum-iron interactions, iron chlorosis was apparent in flax growing in solutions containing 0.1 ppm molybdenum and 10 ppm iron whereas at molybdenum concentrations of 40 ppm chlorosis was not observed.

The possibility that high levels of manganese which might be present in some acid soils would induce or accentuate molybdenum deficiency has been considered. Anderson and Spencer (1) found that a heavy application of manganese sulfate reduced the uptake of molybdenum by flax and induced molybdenum deficiency. However, these effects could have resulted from competition between molybdate and sulfate ions for absorption sites on the plant roots (15). When the effects of manganese and sulfate were considered separately in more recent investigations by Anderson and Arnot (2) and Mulder (13), it was found that high concentrations of soil manganese accentuated molybdenum deficiency and decreased the molybdenum content of plant tissue. Mulder, however, was not able to duplicate this interaction between manganese and molybdenum for all plant species tested when grown in soil or with cauliflower when grown in solution culture.

Because of the contradictions to be found in published data relating to molybdenum-manganese-iron interactions and because of the importance of understanding the factors governing the manganese, iron, and molybdenum nutrition of plants, these further

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studies were undertaken. It was hoped that closely controlled experiments coupled with analyses of plant tissue might lead to a better understanding of the nature of these interactions, particularly in regard to the absorption of iron by plants.

Materials and Methods

The nutrient medium employed was a slightly modified Hoagland's solution described by Johnson et al (8). The following quantities of the essential elements in microgram atoms per liter are present: N, 16,000; K, 6,000; Ca, 4,000; P, 2,000; S, 1,000; Mg, 1,000; Cl, 50; B, 25; Mn, 5.0; Fe, 4.0; Zn, 2.0; Cu, 0.5; and Mo, 0.1. A portion of the nitrogen is provided as an ammonium salt rather than as nitrate. This was effective in reducing changes in pH in the culture solutions in spite of large variations in the amount of plant growth. For example, in the experiment to be reported in table I, the average pH of the cultures in which maximum growth of 3.67 g developed was 4.66 after a 24 day growth period while in cultures with only 0.20 g of growth the pH was 4.53. Therefore, adjustment of pH during the growth period was unnecessary.

A 0.002 M $FeSO_4 \cdot 7\,H_2O$ solution adjusted to pH 3.5 served as the iron source; molybdenum was added as $(NH_4)_6MO_7O_{24} \cdot 4\,H_2O$; and manganese as $MnCl_2 \cdot 4\,H_2O$. Since the experiments were of relatively short duration and the amount of growth insufficient to deplete the nutrients, the solutions were not changed during the experiments. In some cases, several additions of iron were made. This is indicated in the presentation of the individual experiments.

The molar stock solutions providing the major essential elements were purified of manganese contamination by heating in the presence of calcium phosphate and calcium carbonate precipitates as described by Stout and Arnon (14) and of moylbdenum by the copper sulfide co-precipitation method of Hewitt (6). Double distilled water was employed in all experiments and the culture containers as well as all glassware were thoroughly cleaned by soaking in dilute HCl and rinsing with double distilled water as is customary in micronutrient experiments.

Marglobe variety tomatoes were used in all experiments. After germination in one tenth Hoagland's solution of the major elements, 3 seedlings were transplanted to each of the 2 quart polyethylene containers which served as culture vessels. The nutrient solutions were aerated continuously. Plaster of Paris lids served both as covers for the plastic containers and support for the plants. The translucent containers were covered with aluminum foil to eliminate

Table I

Effects of Varying Concentrations of Molybdenum and Manganese Added to the Culture Medium on the Yield, Iron, Manganese, and Molybdenum Content, and the Development of Iron Deficiency Chlorosis of Tomato Plants

Treatment	Added to culture solution, ppm		Ave dry wt of plants per culture, g	Analysis of leaf and stem tissue, ppm			Severity of Fe deficiency chlorosis *
	Mo	Mn		Fe	Mn	Mo	
A	0.7	0.27	2.73	96	45	19	None
B	"	5.4	2.05	60	527	14	None
C	"	10.8	2.09	51	990	16	None
D	"	21.6	0.22	..	...	...	+++++
E	3.35	0.27	3.67	56	31	445	None
F	"	5.4	1.53	29	680	573	+++
G	"	10.8	0.64	33	1148	898	++++
H	"	21.6	0.20	..	...	...	+++++
I	13.4	0.27	2.50	39	41	1796	None ++++
J	"	5.4	0.32	33	932	3404	++++
K	"	10.8	0.30	46	1007	2485	+++++
L	"	21.6	0.15	...	...	...	+++++
M	33.8	0.27	1.11	44	60	4399	+
N	"	5.4	0.45	...	...	...	++++
O	"	10.8	0.29	...	...	...	+++++
P	"	21.6	0.18	...	...	...	+++++
Q	67.0	0.27	0.23	...	...	...	++
R	"	5.4	0.16	...	...	...	+++
S	"	10.8	0.15	...	...	...	+++
T	"	21.6	0.12	...	...	...	+++

The growth period was 24 days. Iron was added (0.22 ppm as $FeSO_4 \cdot 7\,H_2O$) to each culture 6 times during this period.

* The degrees of iron chlorosis associated with these designations are: +, faint, mottled chlorosis on the terminal leaves; ++ and +++, increasing proportion of the terminal leaves chlorotic except the veins which remained green; ++++, the terminal growth was completely chlorotic; +++++, in large areas of the terminal growth the chlorotic tissue had become necrotic. Similar designations are employed in table II.

light and reduce temperature changes. All experiments were carried out in a conventional greenhouse. Every treatment in each experiment consisted of duplicate cultures. The plants from each culture were harvested separately; divided into roots and tops; the roots were washed thoroughly in distilled water; and all samples were dried at 65° C.

To avoid contamination due to grinding, the entire samples were wet-ashed in a nitric-perchloric-sulfuric acid mixture. Iron was determined as an *o*-phenanthroline complex; manganese by oxidation to permanganate; and molybdenum as the amber colored complex of quinquevalent molybdenum and thiocyanate. Each of the duplicate samples from a specific treatment was weighed and analyzed separately. The values reported in tables I and II are averages of these determinations. Of the treatments producing more than 0.5 g growth, in only one did the individual dry weight values vary from the average reported by more than 15 %. Among the chemical analyses, the duplicate determinations for iron in 1 treatment, for manganese in 2 treatments, and for molybdenum in 5 treatments of the total reported exceeded the average values by more than 15 %. The molybdenum analyses, therefore, were considerably less consistent than the iron and manganese.

Experimental Results

Molybdenum-Manganese-Iron Interactions: The initial experiment was an attempt to reproduce with the tomato plant Millikan's results which had shown molybdenum capable of offsetting manganese toxicity in several plant species. Table I shows the treatments tested which included 5 concentrations of molybdenum varying from 0.07 to 67.0 ppm with each level of molybdenum combined with manganese concentrations of 0.27, 5.4, 10.8, and 21.6 ppm. The highest concentrations of molybdenum or manganese were chosen to be within suspected toxicity ranges.

The highest levels of manganese obviously were toxic as shown by the marked, successive decreases in growth in treatments A to D and E to H. If the results agreed with those reported by Millikan, the injurious effects resulting from a specific level of manganese would have diminished as the concentration of molybdenum in the culture solution increased. Exactly the opposite was obtained in all cases. For example, in Treatments B, F, and J all of which contain 5.4 ppm manganese, but 0.07, 3.35, and 13.4 ppm molybdenum respectively, growth decreased from 2.0 to 1.53, and to 0.32 g of dry tissue. Similarly in Treatments C, G, and K containing 10.8 ppm manganese but increasing molybdenum, growth decreased from 2.09 to 0.30 g.

The decreases in growth induced by moderate levels of manganese and accentuated by molybdenum, for example Treatment F in comparison with Treatment J, correlated with observations on the degree of iron chlorosis and indicated that both elements were capable of affecting iron availability. When a leaf which was very chlorotic and much reduced in size from a plant of Treatment G was painted with a ferrous sulfate solution near the conclusion of the experiment, a normal green color was quickly restored, thus substantiating that the observed chlorosis was due to iron deficiency. The significance of the reduced chlorosis associated with the highest concentration of molybdenum (Treatments Q to T) will be discussed in a subsequent section.

With very high levels of manganese (Treatment H) or of molybdenum (Treatment T) additional symptoms not normally associated with iron deficiency were apparent. For manganese toxicity, this consisted of a brown, necrotic spotting and streaking of lower leaves, stems, and petioles; for molybdenum toxicity, a golden pigmentation of the newest growth and extreme stunting of leaf size near the growing point, particularly the basal leaflets.

Where sufficient material was available, the plant tissue was analyzed for iron, manganese, and molybdenum. The results correlate well with the weight data and visual observations of iron chlorosis. Increasing concentrations of manganese in the culture solution resulted in much higher concentrations in the plant tissue and at the same time a decrease in iron content, from 96 to 51 ppm in Treatments A, B, and C and from 56 to 33 ppm in Treatments E, F, and G. The effect of increasing concentrations of molybdenum in the culture medium on the molybdenum and

Table II

Ability of Relatively Low Concentrations of Molybdenum Added to the Nutrient Solution to Induce Iron Deficiency in Tomato Plants

Treatment	Added to culture solution, ppm		Ave dry wt of plants per culture, g		Fe content of tissue, ppm		Mo content of tissue, ppm		Severity of Fe Chlorosis *
	Mo	Fe	Tops	Roots	Tops	Roots	Tops	Roots	
A	0.067	0.11	3.28	0.63	29	34	11	115	+
B	0.67	0.11	2.52	0.72	29	29	54	1195	++
C	3.35	0.11	0.82	0.37	28	99	930	2710	+++
D	6.70	0.11	0.39	0.14	30	171	1270	2785	+++++
E	0.067	1.10	3.17	0.65	55	780	12	48	None

The growth period was 25 days. Iron was added to Treatments A, B, C, and D only at the start of the experiment, but to cultures of Treatment E (0.22 ppm) 5 times during the growth period.

* See table I for explanation of symbols.

iron contents of tissue can be evaluated from the data from Treatments A, E, and I. The molybdenum content increased from 19 to approximately 1800 ppm while the iron content, rather than increasing or remaining constant as might be predicted from Millikan's results, decreased from 96 to 39 ppm.

The possibility that an interaction between molybdenum and manganese might also be observed through the capacity of increasing concentrations of either element to accentuate the symptoms due to a deficiency of the other was investigated. Cultures were prepared from which molybdenum was omitted and manganese varied from 0.27 to 5.4 ppm and from which manganese was omitted but molybdenum varied from 0.067 to 3.4 ppm. There was no indication that increasing levels of either manganese or molybdenum had any effect whatsoever in inducing or accentuating a deficiency of the other element. This is in agreement with results reported by Mulder (13) from nutrient culture experimentation.

MOLYBDENUM-IRON INTERACTIONS: In the experiment reported in table II, the capacity of molybdenum to affect iron availability was further evaluated with manganese added to all cultures at a low concentration and thus omitted as a variable. Except for the control, the concentration of iron employed was relatively low, 0.11 ppm added at the start of the experiment. This resulted in plants which were mildly chlorotic by the end of the growth period in cultures receiving even the lowest level of molybdenum, but growth was not affected as shown by a comparison of yields from Treatment A and the control cultures of treatment E which received a total of 1.10 ppm iron during the course of the experiment. Molybdenum concentrations varied from 0.07 to 6.70 ppm, far below the maximum tested in the initial experiment.

Molybdenum was surprisingly effective in accentuating iron deficiency for although only 0.67 ppm molybdenum was present in Treatment B, top growth decreased to 2.52 g from the maximum of 3.28 g in Treatment A. Further increases of molybdenum reduced growth to 0.82 and 0.39 g in Treatments C and D respectively. These decreases correlated with marked intensification in the terminal chlorosis characteristic of iron deficiency.

Analyses for iron and molybdenum show that in spite of the sharp decreases in growth in Treatments B, C, and D, the iron content of the leaf and stem tissue remained constant at approximately 30 ppm. This would seem to indicate that 30 ppm is the critical iron content for growth under the conditions of this experiment, and that yield was determined by the availability to the plants of sufficient iron to maintain the content of the tops at this level.

The iron content of the roots also was approximately 30 ppm in Treatments A and B. However, with higher molybdenum concentrations in the culture solution in Treatments C and D, the amount of iron rather than remaining constant as in the top growth increased markedly to 99 and 171 ppm.

An attempt was made to determine if the reverse of the effect described above also could be demonstrated, that is if increasing concentrations of iron added to the culture medium would accentuate molybdenum deficiency. When molybdenum was omitted from the culture solution and iron added in concentrations of 0.66, 1.32, and 3.30 ppm, the results presented in table III were obtained. The weights of tissue from the duplicate cultures rather than average yields are included to show that except for one culture of Treatment B, the results definitely show that as the iron concentration in the culture medium increased growth decreased from a maximum of approximately 2.8 g in Treatment A to only 0.2 g in Treatment C. Visible observations indicated these decreases to be due to molybdenum deficiency.

The most likely explanation for the inconsistency in Treatment B would seem to be that one culture had become contaminated with molybdenum. The experiment was repeated employing a wider range of iron concentrations, and during early stages of growth there were visual indications of effects similar to those reported in table III. Unfortunately, these differences disappeared by the conclusion of the experiment and equally severe molybdenum deficiency symptoms were present in all treatments. It may be significant that growing conditions were quite different during the two experiments. In the initial trial, cloudy and cool weather prevailed; during the second, temperatures were very high and sunlight intense. Possibly there is only a relatively slight difference in molybdenum availability associated with varying iron supply and this is reflected in growth differences only if a minimum rate of supply of molybdenum to the top growth is necessary during periods of slow growth.

TABLE III

EFFECTS OF INCREASING IRON CONCENTRATIONS IN THE CULTURE MEDIUM IN ACCENTUATING MOLYBDENUM DEFICIENCY

TREATMENT	CONC ADDED TO CULT SOLN, PPM		OVEN-DRY WT OF TOPS AND ROOTS (G) IN DUPLICATE CULTURES	
	Fe	Mo	A	B
A	0.66	0.00	2.72	2.92
B	1.32	0.00	0.99	7.68
C	3.30	0.00	0.23	0.23
D	0.66	0.01	4.97	6.02

The culture period was 27 days. The indicated concentrations of iron were added to the cultures 3 times during this interval.

DISCUSSION

An increase in the concentration of any constituent of a culture medium to levels far in excess of normal probably will result in essential element interactions

which contribute to reduced growth. These interactions are of significance only when element concentrations reasonably close to those employed in routine culturing of plants are involved as in the present situation in which as little as 0.67 ppm molybdenum accentuated iron deficiency. The nature of this interaction is of immediate interest, particularly because a cation and anion are involved thus making unlikely the possibility of a direct competition in the activation of an iron containing enzyme as sometimes is proposed for iron-manganese antagonisms. Inactivation of the iron through formation of an iron-molybdenum precipitate of very low solubility is a possibility and is supported by the data in table II showing a constant iron content in top growth although root contents increased as higher molybdenum levels were added to the culture solution. Warrington (17) also suggests that molybdenum affects the upward translocation of iron. It is difficult to determine by analyses of the roots whether the proposed iron-molybdenum precipitate adhered to the roots after forming in the culture medium or formed in the roots following absorption of the iron and molybdenum. However, since very low concentrations of the 2 elements are involved (0.11 and 0.67 ppm of iron and molybdenum added to the culture medium) it seems more likely that through absorption and accumulation the concentrations of these elements within the plant are raised to the values reported by Jones (10) to be necessary for ferric molybdate precipitation. The mechanism involved would be similar to that reported by Biddulph (4) for formation of iron-phosphate precipitates in plant tissue which result in reduced iron availability.

It is impossible to evaluate definitely the reasons for the directly contrasting results presented in this paper and those obtained by Millikan. However, a possible explanation is suggested by the data from Treatments Q to T in table I. Cultures in this series received 67 ppm molybdenum, a very toxic concentration as shown by almost complete lack of growth, but the chlorosis observed was less than in cultures receiving much lower concentrations of molybdenum. Apparently the effects of the molybdenum were so severe that after transplanting the seedlings to the culture solutions, symptoms of molybdenum toxicity masked the iron chlorosis or growth ceased before iron chlorosis developed. On the basis of observations alone, this could have been interpreted as a beneficial effect of molybdenum. However, it seems doubtful that practical significance should be attached to this effect unless it results in plant growth comparable to control cultures and is produced by lower concentrations than the 5 to 25 ppm reported by Millikan.

Several possible applications of the results obtained to agriculture are apparent. In the first place, the data indicate that molybdenum application would not be a practical means of alleviating manganese toxicity in very acid soils and probably toxicity due to other metals. However, the capacity of relatively low concentrations of molybdenum to directly affe iron availability might be of importance in alkali soils in which molybdenum availability is at a max mum but iron at a minimum. As reported in tab II, iron chlorosis was associated with tissue conter of molybdenum as low as 54 ppm, and the results Barshad (3) show that the molybdenum content a number of plant species collected from the fie exceeded this value. While the results are inconclu sive the possibility that high levels of iron may accentuate molybdenum deficiency also has been indicated in this paper. This could be a contributing factor to the generally decreased availability of molybdenum to plants in acid soils even though Stout et al (15) have shown that in nutrient cultures decreasing pH promotes the capacity of plants to absorb molybdenum. This effect would be associated with quantities of iron far less than provided in experiments by Jones (9) showing molybdenum availability in soils to be affected by the soil ferric oxide content.

Summary

In direct contrast to results reported by other investigators, molybdenum was found to accentuate iron chlorosis in tomatoes induced by excess concentrations of manganese in the nutrient medium. Tissue contents of iron, manganese, and molybdenum correlated closely with visual observations and growth data, for the iron content decreased with increasing concentrations of either manganese or molybdenum in the external medium. Further experiments on iron-molybdenum antagonism showed that as little as 0.67 ppm molybdenum added to the culture medium induced iron chlorosis and reduced growth. It is proposed that this interaction is due to formation of an iron-molybdenum precipitate of very low solubility in the root tissue which makes iron unavailable to the top growth. The results also indicate that high concentrations of iron may accentuate molybdenum deficiency. The significance of the results in terms of plant nutrition and agriculture are discussed.

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FURTHER STUDIES ON COMPARATIVE MOBILITY OF LABELED HERBICIDES[1, 2]

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The primary object of the work described in this paper was to study comparatively the absorption and translocation of several herbicides. The fate and toxic action will be the subject of future work. Consequently the various labeled molecules were used at the lowest dosage level that would produce satisfactory autographs, in order to keep toxicity at a minimum. For this reason, these labeled materials may be looked on as simply a variety of isotopic tracers employed to discover the inherent differences in behavior of different molecules as they penetrate plant tissues and move in the vascular channels.

We have learned from previous work (5, 10) that different herbicides move in different quantities and at different rates in whole plants and in potato tuber tissue. Low mobility may result from active accumulation by living cells (9) at points of application, or from lack of an active sink. High mobility may result from lack of active accumulation or metabolism at the point of application. It also requires rapid movement of food materials in the plant. The complex interaction of these several variables suggests a detailed study of mobility as a function of age and leaf maturity, and of other factors that regulate food movements in plants.

Some may question the use of radioautographs as evidence for movement of specific compounds pointing out that the compound applied may undergo decomposition or chemical rearrangement during penetration or translocation. It is true that many compounds do, of which urea is a well known example. There is evidence, however, that many of the herbicides are quite stable in plants. For instance, 2,4-D has been shown to persist in plants for months or even for years. In the use of labeled 2,4-D we often find typical formative effects in the regions of high accumulation as shown by autographs. And certain studies are showing that dalapon is very stable in the cotton plant (7). In the present exploratory work, factors responsible for distribution of the labels are under investigation. When these are understood, studies on the fate of the applied chemicals can be pursued on a more logical basis for, from radioautographs, one has an indication of where an applied chemical is located and hence he may sample his treated plants intelligently.

METHODS[3]

Barley, Zebrina, and cotton plants were grown in nutrient solutions in the greenhouse. Tracer solutions of 2,4-D*, ATA*, MH*, IAA*, urea*, and monuron* were made up in 50 % ethyl alcohol con-

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[3] The following abbreviations and trade names are used: ATA, 3 amino,-1,2,4 triazole; dalapon, sodium 2,2 dichloropropionate; IAA, indole acetic acid; MH, maleic hydrazide; monuron,3,(*p*-chlorophenyl)-1,1-dimethylurea; 2,4-D, 2,4-dichlorophenoxyacetic acid. Labeling in all cases was with C^{14}. The asterisk implies "radioactive."

taining 0.1 % Tween 20. Specific activities were adjusted to 0.5 mC per mM for this group. Some 2,4-D* was used at 5 mC per mM.

When plants for an experiment were ready, the leaf to be treated on each plant was marked in the afternoon, and on the following morning the droplets were applied, using a micropipette as illustrated ((12), fig 12). One half hour before termination of the experiment the treated spots were covered with small pieces of masking tape. This avoids contamination during mounting of the plants.

At the termination of the treatment period the plants were removed from the culture jars, the roots quickly rinsed in running tap water and dried with Kleenex tissue, and placed in order in the screen tray of the freeze-dry apparatus. They were quick-frozen with pulverized dry ice, freeze-dried, mounted, pressed, and autographed as described in a previous paper (12).

Results and Conclusions

First to be considered will be a series of barley plants treated at successive stages of growth. Figure 1 shows mounts and autographs of young plants having 3 or 4 expanded leaves. Treated on the 1st, 2nd, and 3rd leaf, these plants translocated appreciable amounts of 2,4-D* into their roots. Treated on the 4th leaf, none of the tracer reached the roots. Dosage was 0.25 μC per plant. Specific activity was 5 mC/mM. Treatment time was 1 day.

Figure 2 shows some older barley plants. Treated with 2,4-D* on leaf number 5, they show very little tracer in the roots. Dosage was 0.5 μC per plant. Specific activity was 5 mC/mM. Treatment time was 1 day. Apparently, as barley plants mature, more and more of the assimilates are utilized in production of new leaves and the inflorescence. However, as will be shown, assimilate movement from the healthy basal leaves into the roots is still taking place, but apparently at a slow rate.

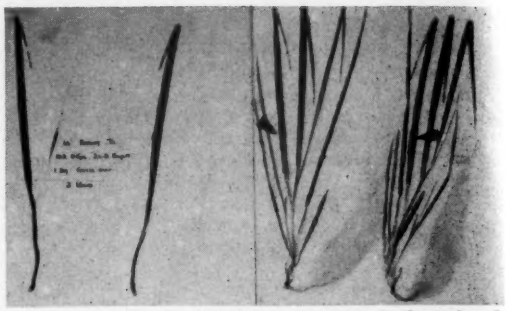

Fig. 2. Older barley plants treated on leaf number 5. Autograph left, plants right. Treatment as in figure 1.

The next experiment involves a time series with 2,4-D* and MH*. These 2 tracers were chosen as examples of the extremes of mobility of phloem-mobile chemicals. Barley plants were used and applications were made to leaf number 2 with 2,4-D* and with MH*. The dosage was 0.05 μC per treat-

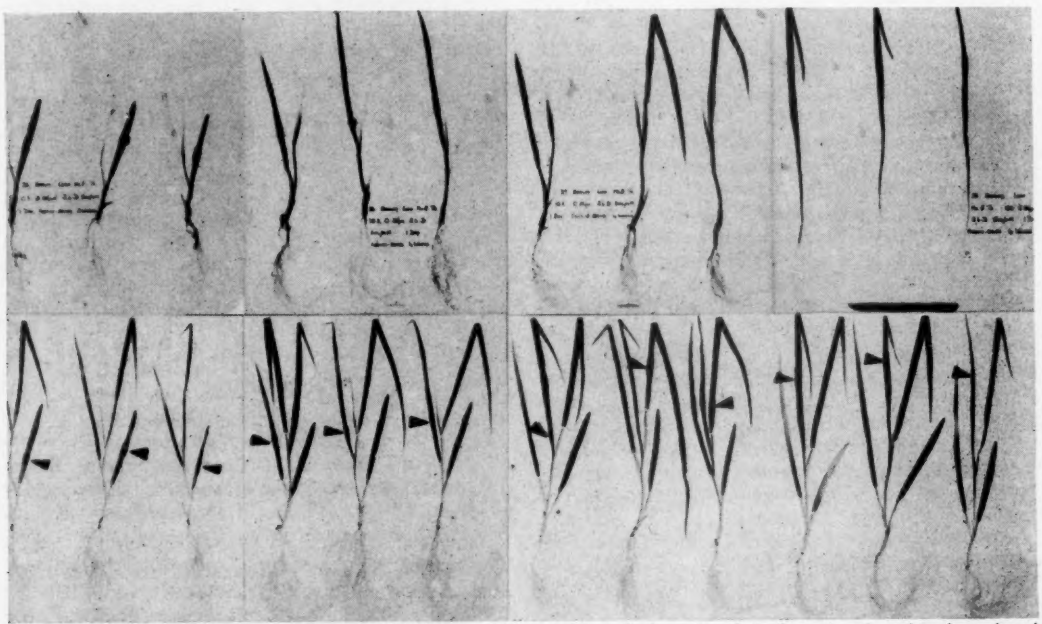

Fig. 1. Barley plants treated with 2,4-D* on leaf number 1, leaf number 2, leaf number 3, and leaf number 4, respectively (left to right). Autographs are at the top, mounted plants beneath. Dosage was 0.25 μC per plant. Specific activity was 5 mC/mM. Treatment time, 1 day.

ment. Specific activity was 0.5 mC per mM. Treatment times were 3, 9, and 27 hours. Figures 3 and 4 present the plants and the autographs. These show that, although the droplets of applied solutions were dry within an hour or less, penetration and translocation of the chemicals continued much past the 9-hour period. The superior mobility of MH* over 2,4-D* is well illustrated.

As previously described (4) much is expected of the plant when 2,4-D is used as a translocated herbicide, for the toxic molecules must move through living tissues in order to develop concentrations in the roots capable of bringing about complete destruction. In this connection rate of penetration is important. If the toxicant enters the leaf rapidly as in the case of the light alkyl esters, contact toxicity destroys the source of the translocation mechanism. And if penetration is too slow insufficient toxicant is absorbed to bring about a complete kill.

From figures 3 and 4, it is apparent that both 2,4-D* and MH* continue to move into leaves long after the droplets have dried. Here the Tween 20 involved in the formulation probably serves as a filming agent to hold the 2,4-D* in intimate contact with the plant cuticle. Further than this it may, by thorough wetting, enable the film to penetrate the external meshwork of the cuticle, provide for collapse of entrapped air bubbles that form a block to penetration, reach the water continuum, and enable the 2,4-D* molecules to establish contact with the outer protoplasmic surfaces of mesophyll cells. In this connection it is interesting to note that in the case of certain difficult-to-control weeds, inclusion of several times the normal content of surfactant has resulted in definite improvement in translocation and root destruction (6).

Having shown the relative differences between 2,4-D* and MH* with respect to transport in the phloem, it seemed desirable to carry on a broader test involving 6 labeled tracers. Because of the great uniformity in performance of the barley plants, replication was reduced from 4 to 2. Chemicals used were 2,4-D*, IAA*, ATA*, MH*, urea*, and monu-

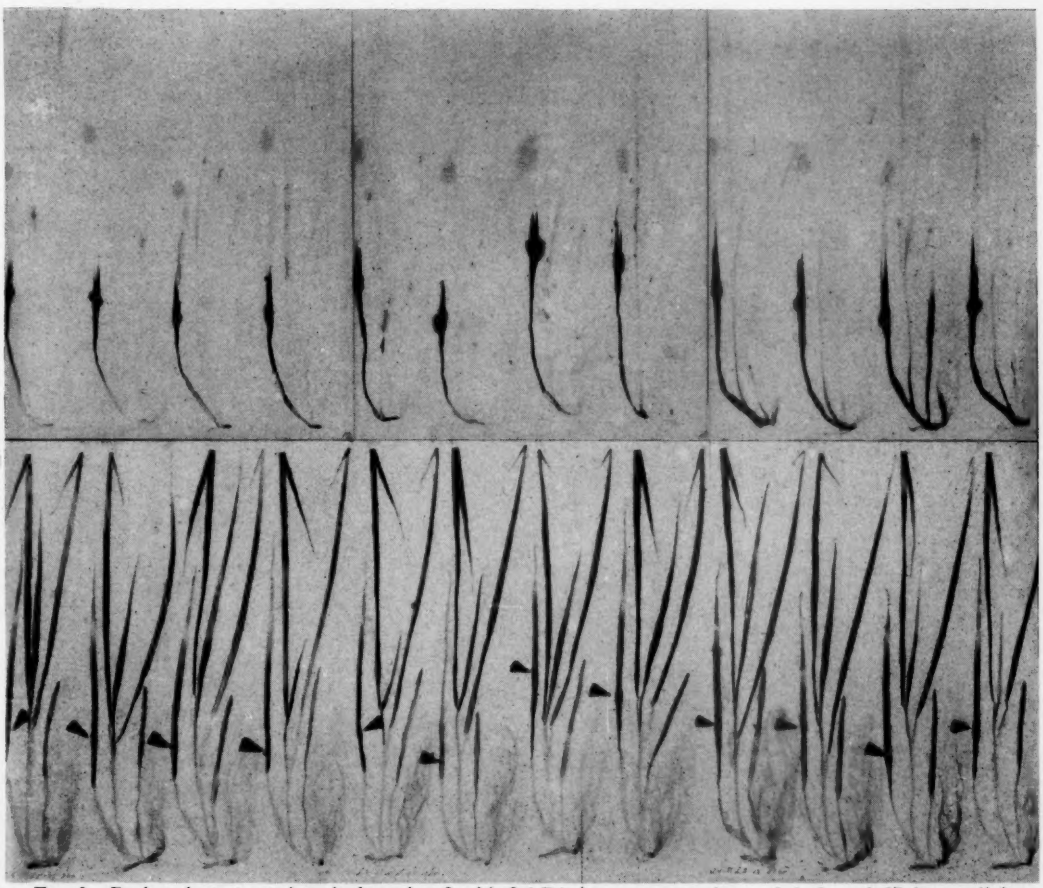

FIG. 3. Barley plants treated on leaf number 2 with 2,4-D* for treatment times of 3, 9, and 27 hours (left to right). Dosage was 0.05 mC. Specific activity was 0.5 mC per mM.

ron*. Dosage was again 0.05 μC. Treatment time was 27 hours on leaves number 1 and 4, respectively.

Figures 5 and 6 show the plants and autographs. They have been arranged in the order given above because the first 4 seem to constitute a series of increasing mobility. Division of labor between leaves 1 and 4 is well illustrated; also the increasing freedom of movement from 2,4-D* to MH*, with IAA* falling 2nd and ATA* 3rd.

Autographs of the urea* treatment are particularly interesting. There is good evidence that this compound is rapidly split during penetration, the CO_2* is immediately synthesized to sugars and amino acids, and in the stem and root, the bulk of the C^{14} is carried as sucrose ((11), Clor, 1958, private consultation). In the autographs of treatment on leaf number 5 it is notable that accumulation is high in the region of the intercalary meristem and again at the root tips; these constitute active sinks for assimilates; between these, movement in the mature tissues takes place, but radioactivity is low.

Monuron* apparently is not split by urease. As in previous cases, it failed to move out of the treated leaves (8). Its movement to the ends of the treated leaves must take place in the apoplast or nonliving phase, along with the transpiration stream from the xylem. This sort of movement has been shown with ATA* and MH* in bean leaves ((12), fig 2).

After these results were obtained, C^{14} labeled dalapon became available and a test similar to the one just described was made, using this material. The barley plants were somewhat larger, so the dosage was increased to 0.1 mC. The plants were so large that only 2 could be mounted on a 10 x 12 sheet; consequently there are 2 sheets for the 1 chemical. Figure 6 shows the mounted plants and autographs. Evidently this chemical moves freely in the phloem; it also transfers to the xylem and moves in the transpiration stream. This is very evident where treatment was on the older leaf. Accumulation in the roots is not so high as occurs with ATA* and MH*.

Because all of the preceding experiments involve

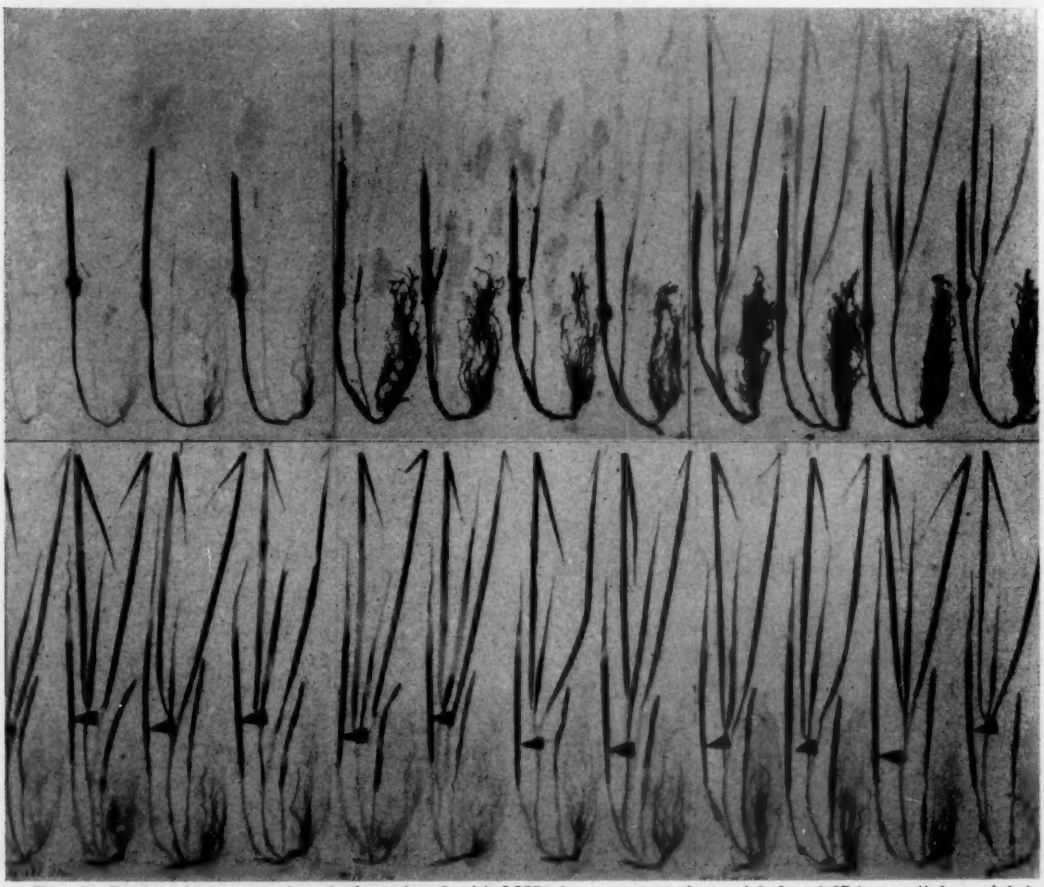

FIG. 4. Barley plants treated on leaf number 2 with MH* for treatment times of 3, 9 and 27 hours (left to right). Dosage was 0.05 μC. Specific activity was 0.5 mC per mM.

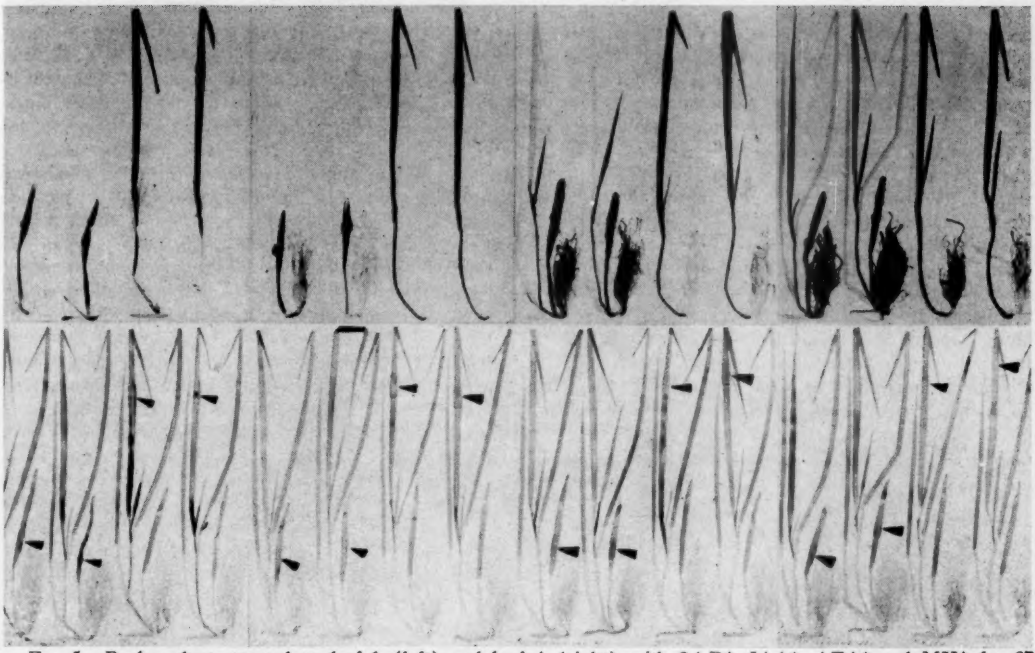

FIG. 5. Barley plants treated on leaf 1 (left) and leaf 4 (right) with 2,4-D*, IAA*, ATA* and MH* for 27 hours (left to right). Dosage and activity as in figure 4.

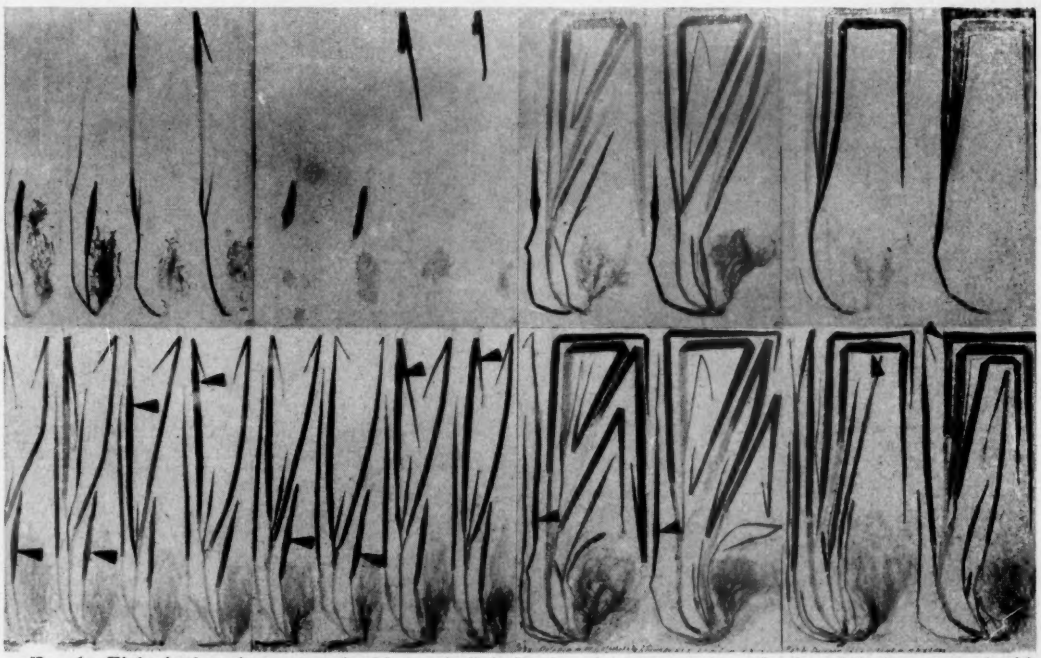

FIG. 6. Eight barley plants treated with urea* (left four) and monuron* (next four) and 4 plants treated with dalapon* (right). Urea* and monuron* treated plants as in figure 3. Plants treated with dalapon* on leaf 3 (left) and leaf 6 (right). Note the low concentration of dalapon* in the roots. Dosage of urea and monuron 0.05 μC; of dalapon 0.1 μC.

a combination of absorption and translocation, it seemed desirable to carry out tests in which these 2 processes could be separated. In order to study absorption alone, a test was set up using cubes of potato tuber tissue. The cubes were cut to a dimension of 1.5 inches and placed on filter paper saturated with water in a closed metal container. A loose lid of cardboard covered the container. Treatments were to 0.25-inch circles on top of the blocks, with lanolin rings to prevent spreading. Times were 2, 4, 8, and 16 days. The same 6 tracers as in the preceding test were used. Dosage was 0.05 μC per treatment with solutions having a specific activity of 0.5 mC per mM. Each chemical was applied to 2 tuber blocks, and at the end of the treatment time 1 block was split vertically and a 2 mm slice through the middle of the treatment circle was taken. The 2nd block had three 2 mm horizontal slices removed, starting on top. The slices were all freeze-dried, mounted, pressed, and autographed. In mounting, the 3 horizontal slices were inverted, so that the autographed faces represent the distribution of the chemicals at levels of 2, 4, and 6 mm in the tuber block. Figure 7 shows the tuber slices and the autographs for the 2-, 4-, and 8-day tests. The vertical slices are on the left, with the 2 mm, 4 mm, and 6 mm slices alongside from left to right. Figure 8 shows the autograph for urea*, monuron*, and IAA* from the 16-day test.

Here we note, in this storage parenchyma tissue, that the same relation of relative mobility holds. First in freedom of movement is MH*, followed by ATA*, IAA*, and 2,4-D*. Urea in this nongreen storage tissue in the dark was apparently split to NH_3 and CO_2* and the latter lost to the atmosphere. Monuron* seemed to move only in cell walls and to

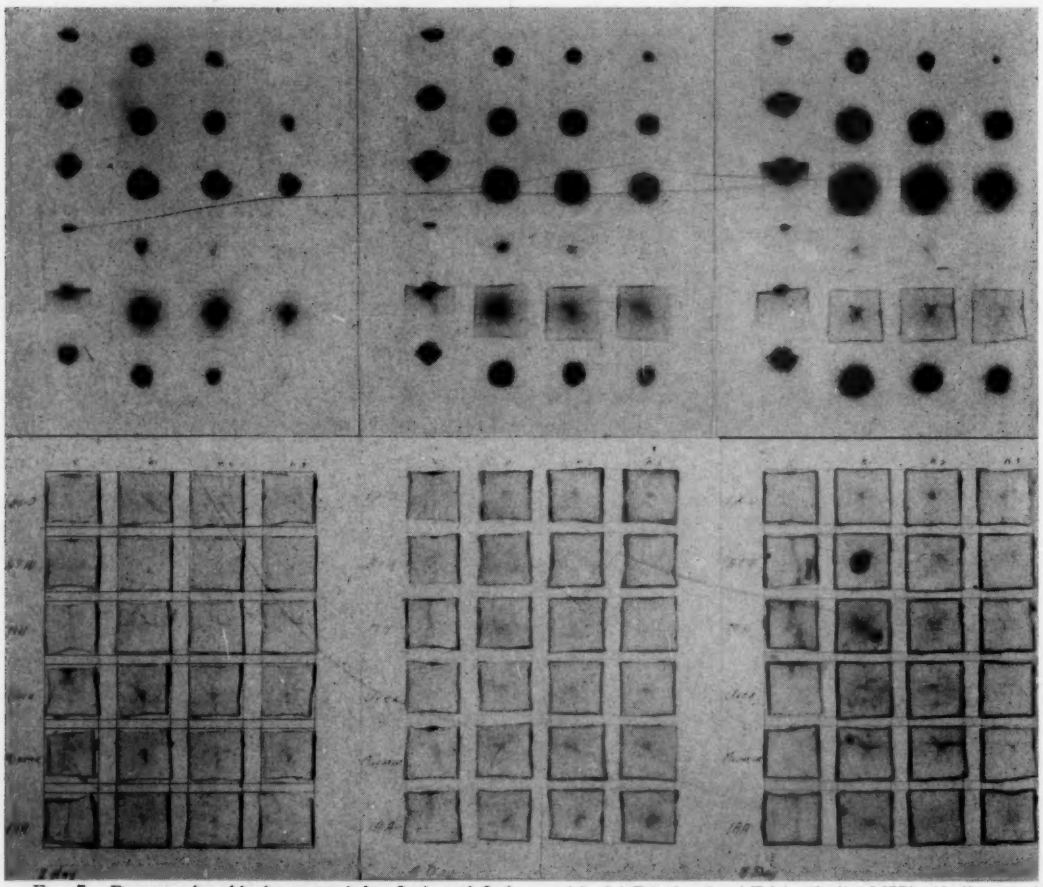

FIG. 7. Potato tuber blocks treated for 2, 4, and 8 days with 2,4-D* (top), ATA* (2nd), MH* (3rd), urea* (4th), monuron* (5th), and IAA* (bottom). Interpretation of these results is given in the text. The dark spots shown in the tuber slices appeared during the remoistening in preparation for mounting and pressing. The slices to the left of each page of mounts are the vertical slices, those in the 2nd, 3rd, and 4th positions from left to right represent the horizontal samples taken at 2 mm, 4 mm, and 6 mm depths from the treated top surface.

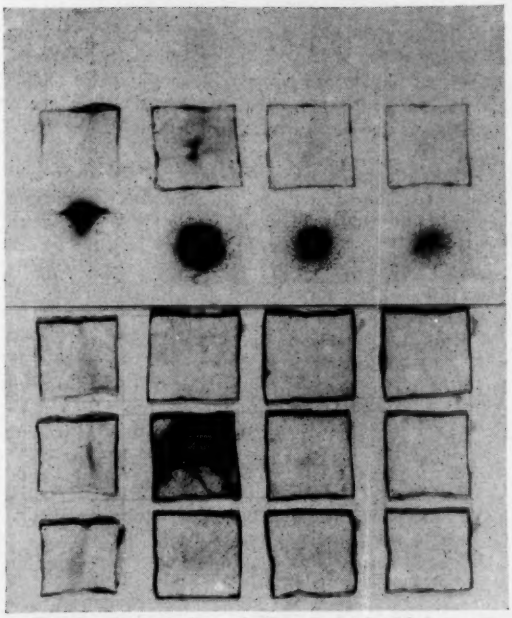

FIG. 8. Autographs and slices from the 16 day treatment. Autographs (top) and slices (bottom). In order starting at the top are urea*, monuron*, and IAA*. Urea was nearly all gone by the 16th day; monuron had diffused along the cell walls and concentrated in the outer cell layers where evaporation was taking place; IAA* was absorbed by the living cells and moved in the phloem strands to a considerable distance beyond the accumulation in storage parenchyma. The black stain in the monuron* slice appeared during remoistening.

concentrate around the edges of the slices, where evaporation was taking place. This is especially notable in the vertical slices, which show increasing concentration in the outer surface from the bottom upward. This follows from the fact that they were virtually standing on a water film from which movement, caused by evaporation, was upward and toward the surface.

DISCUSSION

Evidence has been presented that different herbicide molecules display different degrees of mobility as they move from treated leaves to stems and roots; 2,4-D* tends to stay in the region of treatment, ATA* moves more freely, and MH* moves with great freedom, tending to transfer from phloem to xylem and thus, possibly, to circulate in the plant. Such transfer is indicated by the presence of the tracer in mature, untreated leaves, where it must enter via the transpiration stream. Phloem movement from such leaves is outward as it is from the treated leaf.

Because this same relative mobility is shown in potato tuber tissue, it seems evident that 2,4-D* is accumulated by living cells; cells have a lesser tendency to accumulate ATA*; and MH* moves freely in parenchyma, phloem, and xylem, with little or no tendency to be held in living cells. Evidently, then, 2,4-D* may be held in the chlorenchyma of a treated leaf. If sufficient is applied to saturate the tissues adjacent to the treated spot, some may move on into the phloem, where it is carried rapidly in the assimilate stream. However, here it is subject to withdrawal by phloem parenchyma and available for movement via the symplast to adjacent tissues.

If movement in the phloem is extremely rapid, as in plants having a very active source and sink, then a fair portion of the 2,4-D* that reaches the phloem may move into the roots. However, if photosynthesis, or root growth, or both are sluggish, most of the administered 2,4-D* may end up in parenchyma of the leaf and adjacent stem (fig 2, 3, 5). If the dosage is high, the tracer will move farther, because more tissues may become saturated before movement ceases. And if the treatment time is long, more extensive movement may occur, for 2,4-D*, at least from certain formulations, may continue to penetrate the leaf and move in the phloem for many hours following its application.

Under comparable conditions, ATA* applied to a leaf will penetrate to the phloem and move in the assimilate stream through the stem and into the roots. Because this chemical cannot be utilized in the metabolism of plants it tends to accumulate in root tips where active assimilate utilization is taking place. In Zebrina there was no evidence of transfer from the phloem to the xylem. In barley seedlings some transfer occurred.

MH* apparently moves more freely than ATA*; very little is accumulated along the transport route in the stem; marked accumulation occurs in the root tips and in both Zebrina and barley it transferred to the xylem and mature leaves became marked by the C^{14} (5).

From previous work it seems evident that plants of different age respond differently to 2,4-D* applied to leaves. When this tracer is applied to the cotyledon of the seedling of a dicot, or to the first green leaf of a grass seedling, a fair portion of the absorbed material is moved into the root system. This has also been shown for cotton and cucumber (2) and wild morning glory (3). Treatment on mature basal leaves of older plants results in movement to roots, on upper leaves to shoots, and on young leaves still importing foods no export of tracer occurs (2, 3, 5). These results indicate that any comprehensive study of comparative movement must consider the place of application of the tracers, and age, and the activity of the plants, if consistent results are to be obtained.

Evidently the living parenchyma cells of plants are able to discriminate between various molecules, and some, such as the chlorophenoxy compounds, may be actively accumulated, whereas the cells are relatively indifferent to the presence of others, such as maleic hydrazide. Apparently 2,4-D is not so readily

translocated as formerly described, ((2), p. 287; and (1)). Amino triazole, maleic hydrazide, dalapon and sugars may move much more freely through plants and attain a very general distribution.

It is interesting to note (fig 8) that with passage of time IAA* was accumulated in the phloem strands of the potato tuber and moved beyond the confines of the region of retention by the storage parenchyma. At the other extreme, monuron* seemed not able to penetrate the membranes of living cells, but moved in the apoplast and concentrated in the surface cell walls and in the nonliving xylem vessel walls during the 8- to 16-day period.

An interesting observation is that, as one views the autographs of barley plants through the mobility series 2,4-D*, IAA*, ATA*, MH*, there is increasing evidence of transfer of the tracers from phloem to xylem. Evidently the same molecular characteristics that make for mobility in parenchyma and phloem also make for ready transfer from phloem to xylem. This would seem to indicate that sieve tubes, as well as parenchyma, can accumulate and hold the less mobile compounds and release the more mobile. Because 2,4-D is a more toxic compound than maleic hydrazide, it seems evident that toxicity is not entering into this phenomenon, because injury would presumably make the cells more permeable.

Although the experiments reported here were not designed to solve problems of weed control, they do suggest some very obvious answers to questions raised in the field. The much greater movement into roots of young, as compared with old, barley plants gives a clue to the effect of too early an application of 2,4-D where serious injury to the crop may be experienced. This is simply another reflection of the importance of the source-sink relation in the movement of herbicides. And it is particularly pertinent for 2,4-D, which, because of accumulation, is not one of the better of the translocated materials. The freer movement of amino triazole explains why it is so effective on many weedy plants in the autumn, when the activity of roots may be less than in spring and summer.

The free movement and ready transfer of dalapon in barley may give a clue to its great importance as a killer of perennial grasses. Its persistent systemic distribution may be directly involved in its killing action on these pests.

The relative distribution of 2,4-D* from basal leaves as compared with top leaves, and from the first expanded leaves or cotyledons of seedlings as compared with higher leaves of more mature plants, shows why a lot of low volume application to perennials in the field fails. It seems evident that, if an applied chemical is to move from leaves into the crowns and roots of plants, the lower canopy of leaves must be thoroughly treated. Thorough spraying by ground rig of wild morning glory, Russian knapweed, and a host of woody species has proved essential to control in the field. A hasty pass over such plants with a low volume application by airplane places the chemical on the wrong foliage. It may produce an excellent top kill, but it seldom kills the roots.

Finally, it should be apparent that any screening or testing program that is going to spot translocated herbicides quickly and effectively, should include some simple radioautographic technics that tell where a chemical moves in an intact, healthy plant.

Experimental work described in this paper was done in the Department of Agriculture, University of Oxford, where the writer spent a Sabbatical year. Professor G. E. Blackman kindly provided the equipment and facilities required for the radioautographic work; he and his staff created an especially congenial atmosphere for research. ATA* was provided by Amchem, Inc., monuron* by DuPont, MH* by Naugatuck, dalapon* by Dow. Urea*, 2,4-D* and certain facilities were available through A.E.C. Contract AT(11-1)-34 Project No. 9.

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SOME OBSERVATIONS ON POTASSIUM ACCUMULATION IN CORN ROOT MITOCHONDRIA [1, 2]

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The accumulation of ions by plant tissue has been studied extensively, especially with regard to the kinetics and overall metabolic requirements of the process (19, 26, 28). In order to study the process more precisely, however, a simpler system than the whole tissue has to be used. Owing to the fact that no intermediate product of the process is known, the minimum system in which the accumulation of ions can be studied has to consist of 2 phases separated by a barrier and separately analyzed. The barrier must be impermeable to the diffusion of free ions, but possess a mechanism of ion transport, the hypothetical carrier, and a source of energy to facilitate an endergonic step which makes the process essentially irreversible.

In recent years, mitochondria from various sources, mostly animal, have been used as the minimum system for the study of the exchange and accumulation of ions (2, 10, 11, 21, 27, 31, 32). In general it has been found that in addition to the large amount of exchangeable ions associated with the isolated mitochondria, a certain fraction was tightly bound and could not be exchanged. In most cases the requirement for energy for the binding of the non-exchangeable ions has been recognized, but no attempt has been made to further localize the energy linkage. Uncouplers such as DNP[4] were found to be inhibitory to accumulation, but in the work of Spector (31) and Gamble (10) there is some indication that the effect of DNP was not directly on the process of accumulation but on the retention of the accumulated ions.

A clue as to what constitutes the diffusion limiting barrier is given in the work of MacFarlane and Spencer (21). While studying the nature of the acid phosphatase of rat liver mitochondria, they observed that the enzyme was inaccessible to β-glycerophosphate as long as the mitochondria were intact, but became accessible after lysis or after treatment with *Clostridium welchii* toxin.

From a theoretical point of view, lecithin and other phospholipides have the physical attributes required for a barrier which limits the diffusion of ions. Membranes containing lecithin are impervious to ions but are readily permeable to undissociated molecules (36). This characteristic is necessary in a barrier to prevent back diffusion of ions, while allowing a non-ionic ion carrier complex to diffuse through. Lecithin, moreover, is found in all plants and presumably is a constituent of all plant membranes (30).

In the investigation reported here an attempt is made to relate the oxidative and phosphorylative activities of the mitochondria to ion accumulation by mitochondria. In addition, the importance of phospholipides in maintenance of the diffusion limiting barrier has been studied. The particulate fraction from roots of corn seedlings which sediments between 3,000 and 21,000 × G was used. This is a heterogenous fraction consisting of mitochondria, vesicular elements of the endoplasmic retriculum and some adhering microsome granules (18). For the sake of convenience the preparation will be called "mitochondria" throughout, bearing in mind that the term does not imply morphological homogeneity.

MATERIALS AND METHODS

Roots of 3 day old seedlings of hybrid corn, WF 9 × M 14, were used throughout. The seeds were germinated by a method previously described (14). The roots were ground in an ice-cold mortar with grinding medium containing 0.5 M sucrose, 0.07 M potassium phosphate and 0.005 M EDTA, pH 6.8. After grinding, the slurry was passed through eight layers of cheesecloth and centrifuged for two minutes at 3,000 × G to remove nuclei and debris. The supernatant was centrifuged for 15 minutes at 21,000 ×G, decanted, and the pellet resuspended in 50 ml of grinding medium and resedimented. The washed pellet was taken up in 0.5 M sucrose to the desired volume. All isolation procedures were carried out in the cold.

Respiration was measured manometrically in a Warburg respirometer at 29° C, with air in the gas phase, 0.2 ml of 20 % KOH in the center well and 2.5 ml final volume in the external compartment.

Inorganic phosphate was determined by the method of Fiske and Subbarow (8). Nitrogen was determined by digestion and subsequent Nesslerization.

The respiration medium contained the following final concentrations of additives and was used throughout except for changes specifically noted: 0.010 M K_2HPO_4; 0.2 M sucrose; 0.016 M substrate; 0.022 M glucose; 0.001 M $MgSO_4$; 0.001 M AMP; 4×10^{-4} M thiaminepyrophosphate; 2×10^{-4} M DPN; 4×10^{-5} M CoA; 3×10^{-6} M cytochrome c. 0.1 to 0.3 mg of mitochondrial nitrogen were added to each flask. The final pH was 6.8.

Phospholipide medium consisted of 0.004 M acetylcholine, 0.004 M *a*-glycerophosphate and 0.0005 M CMP in final concentration added to the respiration medium.

[1] Received March 6, 1959.

[2] This report constitutes a portion of a thesis submitted by J. S. Kahn in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[4] Abbreviations used: DNP, 2,4-dinitrophenol; DDC, diethyldithiocarbamate; MeB, methylene blue; TCA, trichloroacetic acid; EDTA, ethylenediamine tetraacetic acid.

POTASSIUM ACCUMULATION: In this work, Rb^{86} was used exclusively as tracer for the potassium ion. This was considered justified in light of the similarity of the two ions and the similarity of behaviour in short term experiments on ion accumulation in plants and microorganisms (6, 14, 20, 25).

Due to technical difficulties experiments on respiration and accumulation by mitochondria were for the most part not conducted concurrently. The accumulation experiments were carried out in 50 ml Erlenmeyer flasks placed on a unidirectional shaker at room temperature (23–25° C). The final volume in the flasks was 5 ml and the medium identical with that for respiration studies. After 30 minutes incubation the flask contents were transferred quantitatively into centrifuge tubes with the aid of 25 ml of cold grinding medium and sedimented by centrifugation at 21,000 × G for 15 minutes. The pellet was resuspended in 25 ml of grinding medium and resedimented. The washed pellet was dissolved in 2 ml of 1 *N* NaOH and aliquots removed for the determination of activity and nitrogen content. Previous experiments (unpublished data) showed that one washing with grinding medium, which contains about 0.12 M potassium, was enough to remove almost all of the exchangeable ions. In order to determine the exchangeable ions which were not washed away, as well as other contamination remaining in the tubes, a sample was carried along in each experiment in which the cold grinding medium was added to the mitochondria and respiration medium at zero time. The value of this sample (20–30 % of control) was subtracted from that of all the treatments.

To maintain anaerobic conditions during incubation, tank nitrogen was bubbled through the stoppered flasks. The nitrogen, however, was not free from traces of oxygen even after passing through a solution of reduced methylene blue and powdered zinc, as it was able slowly to reoxidize methylene blue in the reaction flasks.

Potassium accumulation in the whole root tissue was carried out by the method previously described (14).

The accumulation by mitochondria in situ was determined by incubating the whole roots in labeled potassium solutions. At the end of the incubation the roots were removed, rinsed and a sample taken for the determination of the total activity. The rest of the roots were ground, the mitochondria isolated, washed and the radioactivity determined as above.

Inhibitors were prepared in stock solutions except for KCN which was made up daily. In order to avoid the loss of cyanide by volatilization, the accumulation experiments were carried out in stoppered flasks. In manometric work, 1 M KCN was substituted for the KOH in the center well as suggested by Laties (16). This is supposed to keep an approximate concentration of 10^{-3} M in the vessel.

Ascorbate was determined by titration of a sample of the supernatant solution from TCA precipitation before and after incubation, using the 2,6-dichlorophenol-indophenol method (5).

PHOSPHOLIPIDE DETERMINATION: For estimation of total phospholipides the mitochondria were precipitated and twice washed in 5 % TCA. They were then extracted 3 times with 3 ml of ethanol: ether: chloroform (2:2:1). After evaporation of the solvents from the extract and digestion with H_2SO_4, the phosphate was determined by the method of Fiske and Subbarow.

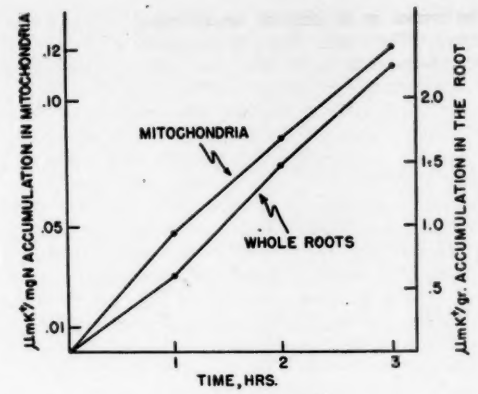
An adaptation of the extraction method of Weiss, et al (37) was used in following the labelling of lecithin with P^{32}. The TCA precipitated mitochondria were extracted 3 times with 4 ml of absolute ethanol and pooled in Folin-Wu tubes. Four ml of CCl_4 were then added to the extract and thoroughly mixed. Inorganic and water soluble organic phosphate, as well as the ethanol were removed by washing the extract 3 times with 15 ml of 1 M KCl and twice with water. The total phosphate and P^{32} activity were then determined on the evaporated and digested CCl_4 extract.

Separation of extracted phospholipides was done by paper chromatography by the method of Witter, et al (38), using a solvent mixture of 2,6-dimethyl-4-heptanone: acetic acid (30:7). The R_f values obtained with standard cephalin and vegetable lecithin were always slightly lower than those reported by Witter.

RESULTS AND DISCUSSION

Initial experiments were designed to find out to what extent the mitochondria will accumulate potassium in situ. Figure 1 shows that the rate is linear over a 3 hour period, as is the rate for the whole root tissue. Considering the average mitochondrial nitrogen of these roots to be about 0.2 mg/gm of fresh weight (18), the rate of accumulation into the mitochondria was about 1 % of that for the whole root. The addition of 10^{-4} M DNP to the roots resulted in a 70 % inhibition of the accumulation by the whole roots as well as by the mitochondria in situ.

The absolute level of non-exchangeable potassium in the mitochondria at the beginning of incubation, as well as the degree to which this initial potassium was lost during incubation was estimated by germinating the seedlings in 0.002 M KCl labeled with Rb^{86}. The radioactivity of the isolated mitochondria was subsequently determined before and after 30 minutes of incubation in respiration medium. Although these seedlings were germinated in KCl, instead of the usual $CaCl_2$, the level of non-exchangeable potassium in the isolated mitochondria was only 0.12 μmole/mg N and half of this was lost to the solution during the 30 minutes of incubation. The amount of this non-exchangeable potassium was very small, comprising only 30 % of the amount which isolated mitochondria will accumulate under favorable conditions in vitro during 30 minutes of incubation (see below). It has to be emphasized that this amount comprises only those ions which were tightly bound and could not be removed by washing and exchange. The total amount of potassium must have been much greater,



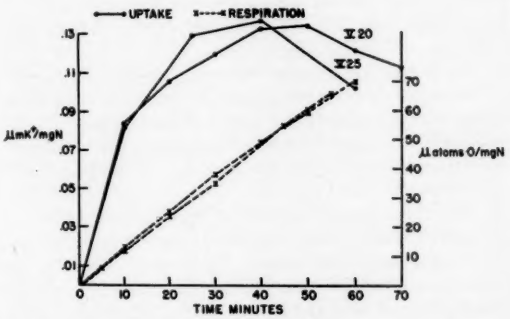


FIG. 1. Time-course of potassium accumulation in excised corn roots, and in mitochondria in situ, from a KCl solution labeled with Rb^{86}. Values for the whole root are per gram fresh weight.

FIG. 2. Time-course of respiration and potassium accumulation by isolated corn root mitochondria. The mitochondria were incubated in respiration medium labeled with Rb^{86}. Two experiments.

but the amount of exchangeable ions was not considered pertinent to the problem here.

Studies on the time-course of accumulation in the isolated mitochondria disclosed that the rate was not linear but rather, decreased with time. The maximum level of potassium in the mitochondria was reached in about 40 minutes of incubation, after which time the mitochondria started to lose the accumulated ions (fig 2). The time-course of respiration is also plotted in figure 2. The rate is essentially constant for one hour. The important point, however, is that the lowest ratio of oxygen consumed to potassium accumulated is over 100, and that no constant stoichiometry exists between the two processes.

These initial studies also showed that when corn root mitochondria are incubated in a respiration medium, 10 to 30 % of their lipide phosphate is lost in one hour. In three time-course experiments the average loss of lipide phosphate was 2.9, 4.5 and 6.3 % at 5, 10 and 20 minutes of incubation, respectively.

The mitochondria exhibited considerable variation in activity from day to day. The average respiration rate was 489 μl O_2/hr/mg N and varied from 332 to 783. The average rate of potassium accumulation was 0.67 μM K/hr/mg N and varied from 0.1 to 1.3 while the loss of lipide phosphate varied from 8.6 to 37 % per hour. In order to study the effects of various treatments on respiration and potassium accumulation with this day to day variation it was necessary to include a standard control treatment with *a*-ketoglutarate as substrate and a potassium concentration of 54 μM/ml in all experiments. Relative to this control treatment, the effects of the experimental additives were quite reproducible and the results are therefore reported on a percentage basis taking the standard control as 100 %.

The rate of phosphorylation was somewhat low for *a*-ketoglutarate (fig 3), and the addition of fluoride did not increase it.

The addition of hexokinase was found to be without effect on either respiration or phosphorylation. This agrees with the observation of Saltman (29) that a large share of the hexokinase in plants can be found with the mitochondria upon isolation.

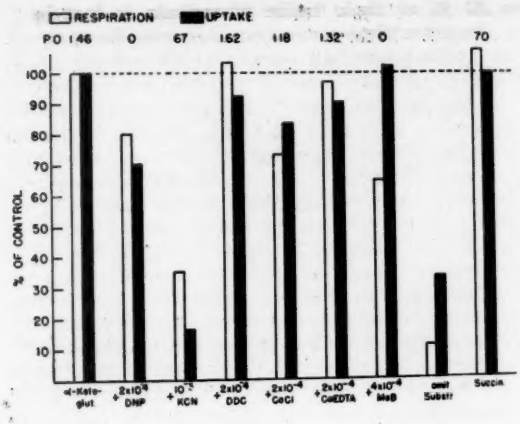
LINKAGE BETWEEN RESPIRATION AND POTASSIUM ACCUMULATION: As mentioned above DNP strongly inhibits potassium accumulation in root tissue, as well as in mitochondria in situ. When added to the isolated mitochondria, however, 2×10^{-4} M DNP inhibited respiration and potassium accumulation to a small degree only, while inhibiting phosphorylation completely (fig 3). It appeared that potassium accumulation was dependent on respiration and that high energy phosphate was not involved and did not serve as energy donor. To check this possibility further, experiments were conducted in a nitrogen atmosphere with a continued slow addition of ATP during incubation to a final amount which was equal to that

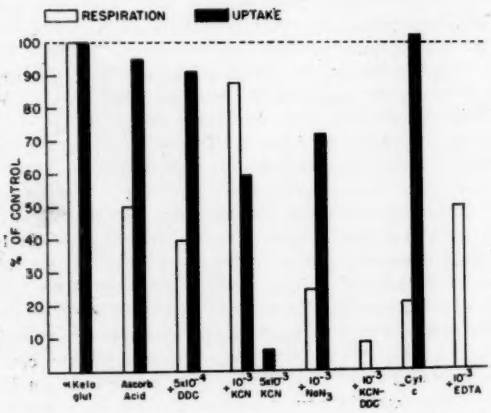
TABLE I

AVERAGE VALUES FOR POTASSIUM ACCUMULATION BY CORN ROOT MITOCHONDRIA, UNDER A NITROGEN ATMOSPHERE

TREATMENT	AVERAGE ACCUMULATION μ MOLES K/MG N/HR	% OF CONTROL
Control*	0.63	100
Under N_2	0.22	35 ± 7
Under N_2 + 20 μm ATP	0.24	38 ± 5
Under N_2 + 4×10^{-4} M MeB	0.13	21 ± 2

* Control was a preparation in respiration medium in air with *a*-ketoglutarate or substrate.





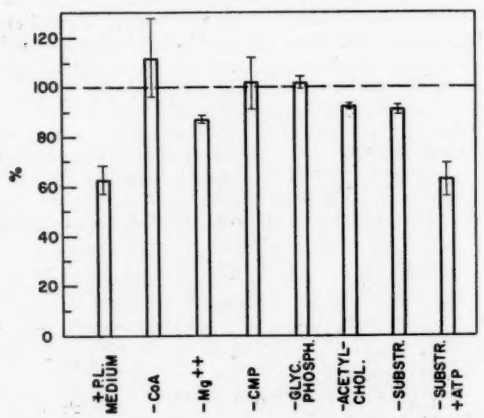


FIG. 3. Average values for respiration, potassium accumulation and phosphorylation by corn root mitochondria under various treatments. Respiration and accumulation are expressed as percent of control. Control was a preparation in respiration medium with *a*-ketoglutarate as substrate. Concentrations of additives are molar. Succinate, when substituted for *a*-ketoglutarate was used at the same concentration (0.016 M).

expected to be formed in an identical vessel under aerobic conditions. The results (table I) supported those obtained with the DNP, in that the added ATP did not increase the potassium accumulation.

James (12) found that in barley seedlings ascorbic acid oxidase was the major terminal oxidase during certain stages of growth, and that the respiration was highly sensitive to DDC. In the corn root mitochondria, however, 2×10^{-4} M DDC had no effect on either respiration or potassium accumulation (fig 3). In contrast, 10^{-3} M KCN was very inhibitory, affecting potassium accumulation more than respiration. In the absence of substrate (fig 3), 11 % of the respiration was sufficient to give 30 % of the accumulation, showing a high efficiency compared to the control.

In intact corn roots, calcium ions accelerate potassium accumulation (14) (the so-called Viets effect (34)). Calcium, when added to the respiration medium, served only to slightly inhibit respiration, phosphorylation and potassium accumulation by the mitochondria. This effect could be reversed by the addition of EDTA (fig 3).

To determine whether electron transport through DPN was involved in potassium accumulation, succinate was substituted for *a*-ketoglutarate as substrate. Although the rate of respiration was a little higher, no change in the rate of potassium accumulation could be observed.

All these results indicated that the rate of respiration by isolated mitochondria was not rate limiting for potassium accumulation. To verify this the effects of methylene blue under both aerobic and anaerobic conditions were compared, using *a*-ketoglutarate as substrate. Under aerobic conditions (fig 3), MeB inhibited phosphorylation completely and respiration by about 40 %. Potassium accumulation, however, was not affected. When, on the other hand, nitrogen was bubbled through the system, thus limiting the oxygen supply, respiratory energy became

FIG. 4. Average values for respiration and potassium accumulation by corn root mitochondria with ascorbate as substrate (0.016 M) expressed as percent of the control. Control was a preparation in respiration medium with *a*-ketoglutarate as substrate. Concentrations of additives are molar. Accumulation in the presence of EDTA was not determined.

FIG. 5. The loss of lipide phosphate from mitochondria during 1 hour incubation. The loss from the control was arbitrarily set at 100 %. The control was a preparation in respiration medium with *a*-ketoglutarate as substrate. P. L. medium: phospholipide medium (see text). The average loss of lipide phosphate from the control was 17.8 % of the initial amount.

the rate limiting factor for accumulation (table I). In this case methylene blue, serving as an electron shortcut from flavin to oxygen, competes for the limited amount of oxygen present. A reduction in electron transport through the cytochrome system can thus reduce the energy available for accumulation, but only when the rate is quite low.

This competitive effect of MeB on potassium accumulation implicates the cytochrome system in the accumulation process. To localize the linkage within narrower limits, the capacity of cytochrome c oxidase to serve as an energy donor was tested next by using ascorbate as electron donor.

The results are summarized in figure 4. Although the rate of ascorbate oxidation was only half that of *a*-ketoglutarate, the rate of potassium accumulation was the same. However, no detectable phosphorylation occurred with ascorbate as substrate. To eliminate the possibility that the oxidation of ascorbate was due to an ascorbic acid oxidase, DDC was added, with no effect on accumulation and only a slight one on respiration, probably due to the weak inhibitory effect of DDC on cytochrome oxidase (13). Another test for the absence of ascorbic acid oxidase is the requirement for added cytochrome c (17). When cytochrome c was omitted from the respiration medium, the rate of ascorbate oxidation was greatly reduced but the rate of potassium accumulation was not affected. The residual oxidation probably due to the endogenous cytochrome c was sufficient to supply the necessary energy. The respiration of *a*-ketoglutarate was not affected by the omission of cytochrome c. The addition of EDTA, which is known to increase the activity of ascorbic acid oxidase (9), had no effect on respiration.

The effect of KCN on the oxidation of ascorbate was peculiar (fig 4). While serving as a powerful inhibitor of accumulation, as it did in the case of *a*-ketoglutarate oxidation, it greatly increased the rate of oxidation. This increase, however, is non-enzymatic, being stable to boiling for 10 minutes. It was also sensitive to DDC and did not require added cytochrome c. Similar phenomena have been observed before (35). Sodium azide did not show the same anomaly, inhibiting both respiration and accumulation (fig 4).

In a number of plants a system of terminal oxidation has been observed which involves the transfer of electrons from DPNH via ascorbate to ascorbic acid oxidase and oxygen (1, 22). These systems require trace amounts of ascorbate for maximal activity. The results in table II indicate that such a system is not operative in the corn root mitochondria, since substrate levels of ascorbate are required.

A concomitant determination of the ascorbate used during respiration gave a value of 0.8 for $\frac{\text{oxygen consumed}}{\text{ascorbate consumed}}$, indicating that the ascorbate underwent a one step oxidation.

Every few experiments a determination of the non-enzymatic oxidation of ascorbate was made by omitting the mitochondrial preparation. Special care was taken to determine that the cytochrome c did not become autooxidizable. In no case did the non-enzymatic oxygen consumption amount to more than 5 % of the respiration of the control, and in most cases was totally undetectable.

TABLE II

EFFECT OF ASCORBATE CONCENTRATION ON RATE OF RESPIRATION AND POTASSIUM ACCUMULATION*

CONCENTRATION	% OF $Qo_2(N)$	% OF ACCUMULATION*
No substrate	11	27
0.002 M ascorbate		43
0.004 M ascorbate	39	
0.008 M ascorbate	46	
0.016 M ascorbate	56	94
L S D 5 %	7	14

* Expressed as percent of *a*-ketoglutarate control.

PHOSPHOLIPIDE SYNTHESIS AND MAINTENANCE OF MITOCHONDRIAL ACTIVITY: Inasmuch as the mitochondrial system of potassium accumulation was unstable (fig 2) and this instability seemed correlated with a loss of phospholipide, an attempt was made to resynthesize mitochondrial lecithin. Attempts to reverse the phospholipide degradation by the addition of CMP and acetylcholine to the respiration medium were without success. Acetylcholine was used because of its ready conversion into phosphorylcholine in the presence of ATP and CoA (4). When, however, *a*-glycerophosphate was added together with the CMP and acetylcholine, giving the complete phospholipide medium, the loss in lipide phosphate was reduced by one-third (fig 5). This effect was strictly dependent on the presence of the three additives, as well as Mg^{++} and CoA. Moreover, ATP could be substituted as an energy source for that formed in respiration. The concentration of acetylcholine and glycerophosphate used had to be kept low since they were inhibitory to the rate of respiration (table III), on which CMP had no effect. Fatty acids were not added to the medium, and it is possible that the reversal of degredation could have been somewhat increased by their addition.

In order to determine whether the decreased loss in lipide phosphate was actually due to synthesis, rather than prevention of degredation, P^{32} was added

TABLE III

EFFECT OF COMPONENTS OF PHOSPHOLIPIDE MEDIUM ON RATE OF RESPIRATION OF CORN ROOT MITOCHONDRIA

TREATMENT	$Qo_2(N)$
Control	379
+ acetylcholine	337
+ glycerophosphate	301
+ phospholipide medium	355
L S D 5 %	27

to the medium. It was expected that the ATP in the system would be labelled with P^{32}, and this in turn would label the phosphocholine which is formed from the acetylcholine (4); the phosphate moiety on the phosphocholine is incorporated into the lecithin (15). In a large number of experiments conducted with inorganic P^{32}, however, only very little of the activity was ever incorporated into the lipide phosphate, at the most 10 % of the amount expected from the differences in total lipide phosphate (table IV). There was, however, an inverse correlation between lipide phosphate content and labeling; the mitochondria

TABLE IV

EFFECT OF VARIOUS ADDITIVES ON DEGRADATION OF MITOCHONDRIAL PHOSPHOLIPIDES AND ON P^{32} LABELLING OF LIPIDE PHOSPHATE DURING INCUBATION

TREATMENT	μMOLE LIPIDE PHOSPHATE LOST	μMOLE P^{32} INCORPORATED
Control	0.43	0.006
+ phospholipide medium	0.27	0.015
— CMP	0.34	0.009
— glycerophosphate	0.36	0.003
— acetylcholine	0.35	0.003

incubated in phospholipide medium lost less lipide-phosphate while gaining the highest degree of labeling.

The possibility existed that the phosphate incorporated originated from the glycerophosphate added, although the work of Mazelis and Stumpf (23) indicated that this was unlikely. This possibility was tested by substituting glycerol for glycerophosphate, and by adding glycerophosphate-P^{32}. Both experiments gave completely negative results; glycerol would not substitute for glycerophosphate in maintaining phospholipide, and no significant amount of P^{32} was incorporated when glycerophosphate-P^{32} was used.

In the course of these experiments it was observed that the washed mitochondria contained an internal pool of inorganic phosphate (about 0.5 μM/mg N) which could not be removed by repeated washing. The possibility that this internal pool was preferentially incorporated into the lecithin was investigated. Washed mitochondria were incubated in a respiration-phospholipide medium labelled with inorganic P^{32} for 45 minutes, after which they were reisolated by centrifugation. The mitochondria were then incubated for 30 minutes in an identical unlabelled respiration-phospholipide medium. Samples were taken before and after the second incubation for phospholipide analysis. The results are given in table V.

During the second incubation the total lipide phosphate decreased, although less than usual due to the continued presence of phospholipide medium and since a good share of the more labile lipide phosphate was already lost during the preincubation. Both the total

TABLE V

CHANGES IN TOTAL LIPIDE PHOSPHATE AND P^{32} LABELLING OF LIPIDES OF MITOCHONDRIA DURING 30 MINUTES INCUBATION IN P^{32}-FREE MEDIUM*

μMOLE LIPIDE PHOSPHATE LOST	μMOLE P^{32} IN LIPIDE		SPECIFIC ACTIVITY	
	BEFORE	AFTER	BEFORE	AFTER
0.027	0.0204	0.0233	0.0237	0.0279

* Mitochondria were pre-incubated for 45 minutes in respiration medium labelled with P^{32}.

P^{32} labelling and the specific activity increased, however. The result indicates that the mitochondria may preferentially utilize phosphate from an internal pool for the synthesis of lecithin. The labeling of the internal pool, however, would appear to lag behind any synthesis of phospholipide. Indeed, the internal pool may consist of a precursor other than orthophosphate. Such an internal pool of slowly labeled phosphate and nucleotides has been described by Pressman (24).

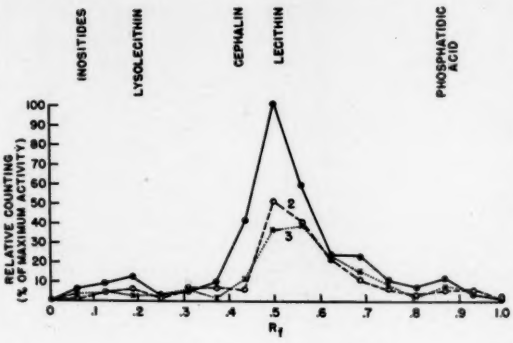
To verify that the P^{32} was actually incorporated into lecithin, the phospholipides extracted after incubation for one hour in a P^{32} labelled medium were separated by paper chromatography. The results are given in figure 6. The bulk of the phosphate incorporated into the lipide appeared as lecithin, with only trace amounts of what is probably inositol phosphatides (R_f 0-0.1), lysolecithin (0.2) and phosphatidic acid (0.85).

As shown in table III, the components of the phospholipide medium had some inhibitory effect on the rate of respiration. The effect was particularly noticeable with only one of the components at a time, indicating that the complete medium had some beneficial effect which was masked by the inhibitory effects of the individual additives. The most obvious source of the beneficial effect would be in the maintenance of the basic membranous structure of the mitochondrion. This should in turn be reflected in other mitochondrial functions. The effect of the phospholipide medium on the time-course of potassium accumulation is given in figure 7. The presence of phospholipide medium markedly increased the rate of K accumulation even in the first 10 minutes. Table VI shows that all the components of the phospholipide

TABLE VI

EFFECT OF COMPONENTS OF PHOSPHOLIPIDE MEDIUM ON ACCUMULATION OF POTASSIUM BY CORN ROOT MITOCHONDRIA

TREATMENT	μMOLE K/MG N/HR
Control	0.193
+ phospholipide medium	0.279
— acetylcholine	0.176
— glycerophosphate	0.198
— CMP	0.229
L S D 5 %: 0.021	



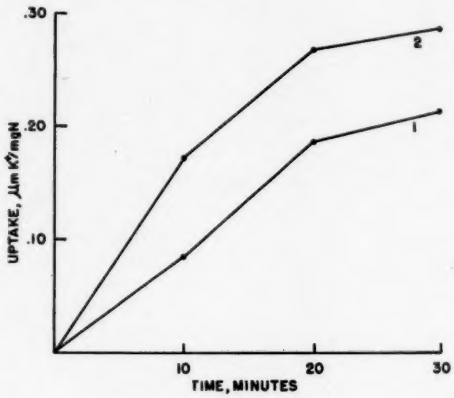


FIG. 6. A paper chromatogram of phospholipides from corn root mitochondria incubated in a respiration medium with inorganic P^{32}. 1) With added phospholipide medium (see text); 2) the same, but substituting glycerol for glycerophosphate; 3) control without phospholipide medium. Average of four experiments.

FIG. 7. Time-course of potassium accumulation in corn root mitochondria incubated in a respiration medium. 1) Control. 2) With added phospholipide medium.

medium were required in order to obtain the increase in potassium accumulation. The need for CMP was never as critical as for the other components, indicating that the washed mitochondria contained small amounts of endogenous cytidine nucleotides.

The possibility existed that the effect of the phospholipide medium in maintaining the level of lipide phosphate was not directly related to the accumulation of potassium, but was due to some other reaction mediated by the same components. It seemed improbable that the actual process of lecithin synthesis was involved in K accumulation since ATP could serve as a source of energy for lecithin synthesis but not for potassium accumulation. In order to more closely discriminate between the effects of the process and the product, mitochondria were preincubated with and without the phospholipide medium, after which the mitochondria were reisolated and their capacity for respiration, phosphorylation and potassium accumulation determined in the absence of phospholipide medium.

The results of the preincubation experiments are summarized in table VII. The rate of respiration decreased during the preincubation, and this effect could not be reversed by the synthesis of lecithin. The loss in phosphorylating efficiency due to the preincubation was completely prevented by the phospholipide medium. As for potassium accumulation, the loss incurred due to preincubation was reversed with the phospholipide medium to about the same extent to which this medium reverses the loss of phospholipides.

Taken as a whole these experiments indicate the following characteristics of potassium accumulation by the mitochondrial system. Accumulation in this instance must be defined as the active penetration of ions into a non-exchangeable phase, irrespective of the concentration gradient. It is considered that the uptake is achieved by the same mechanism whether or not the absorption is against a chemical gradient (33).

1) Potassium accumulation is linked to aerobic respiration, but no constant stoichiometry exists between oxygen consumption and ion uptake. The accumulation process appears to be saturated with respect to respiratory energy at fairly low levels of respiration.

2) The direct involvement of high energy phosphate in accumulation cannot be demonstrated. Dinitrophenol inhibits uptake only slightly and ATP will not serve as an energy source for accumulation. Ascorbate oxidation does not produce high energy phosphate, but does energize accumulation. These observations together with the inhibitions noted with cyanide and azide suggest that the accumulation is probably linked to electron passage through cytochrome a and a_3.

3) The maintenance of lecithin in the membrane is essential to the retention of accumulated ions. The fact that there is a close correspondence between the restoration of potassium accumulation and the synthesis of lecithin gives support to the hypothesis that

TABLE VII

EFFECT OF PREINCUBATING MITOCHONDRIA IN RESPIRATION MEDIUM WITH OR WITHOUT PHOSPHOLIPIDE MEDIUM

TREATMENT	Qo_2(N)	P:O	ACCUMULATION μMOLE K/MG N/HR
No preincubation	536	1.65	0.73
Preincubation with phospholipide medium	423	1.61	0.60
Preincubation without phospholipide medium	404	1.41	0.51
L S D 5 %	60	0.15	0.02

lecithin is part of the diffusion limiting barrier. The capacity to accumulate and retain ions thus seems to be a very sensitive measure of the integrity of the mitochondria and especially of mitochondrial membranes. The limited maintenance obtained was also sufficient to restore oxidative phosphorylation but had no significant effect on respiration.

The postulate that lecithin metabolism can serve as a carrier mechanism (3) is not supported by the preincubation experiments, where the lecithin was synthesized prior to measuring salt uptake. Furthermore, the lecithin metabolism proposed to account for ion accumulation is not in accord with the synthetic system described by Kennedy (15) and supported here.

4) It is doubtful if the mitochondria can serve as carriers in ion accumulation, as has been suggested (7). The suggestion arose from electron micrographs of whole cells in which the mitochondria sometimes seemed to become part of membranes or even to protrude through them to the other side. Such a function seems impossible in the mitochondria described here, considering their relatively low level of potassium (1 % of the whole root) and the slow rate of labeling. If the mitochondria were to serve as ion carriers, they would have to reach a steady-state level of potassium very rapidly, and would not continue to increase their potassium level linearly with time (fig 1). This obviously does not eliminate the possibility that the exchangeable ions on the mitochondria are in some way associated with the carrying mechanism as envisaged by Davies.

5) Studies of accumulation by isolated mitochondria may not be directly applicable to accumulation in intact cells. A case in point is the discrepancy in the effect of DNP on accumulation in the whole tissue and the mitochondria in situ as compared to the isolated mitochondria. That the mitochondria were inhibited in situ is not surprising, considering that the concentration of ions in their environment was reduced due to the reduced accumulation by the cell as a whole. Moreover, roots accumulate DNP very rapidly, so that the internal concentration of DNP surrounding the mitochondria is unknown.

The inhibition of accumulation by DNP in the whole tissue raises another problem. The sites of respiratory energy production in the cell are localized in the mitochondria. For accumulation into the vacuole in the whole cell some sort of energy coinage has to pass from the mitochondria to the tonoplast. The question thus arises whether the accumulation across the tonoplast requires ATP, a different energy source than that required for accumulation into the mitochondria, or whether the linkage is through a soluble compound analogous to ATP as an energy coinage but not involving high energy phosphate. The effect of DNP on the accumulation of the whole root would suggest the first alternative. It is, however, hard to conceive that a different mechanism exists in the cell for accumulation across the tonoplast and for accumulation into the mitochondria.

The experiments with lecithin synthesis may provide an alternative explanation for the contradictory results with DNP. The inhibition of phosphorylation will prevent lecithin synthesis resulting in a net degradation of the ion retaining barrier, but need not directly interfere with the ion accumulation mechanism.

Summary

The linkage of respiration to the accumulation of potassium labeled with Rb^{86} was studied in mitochondria isolated from roots of three-day old corn seedlings. Accumulated ions were defined as those which could not be removed by washing with nonlabeled potassium.

Mitochondria in situ accumulate about 1 % of the total ion accumulated by root tissue. This accumulation is inhibited by dinitrophenol.

The isolated mitochondria showed no constant stoichiometry between respiration and potassium accumulation. The ratio of microatoms oxygen consumed to micromoles potassium accumulated varied from 10 to over 100.

The accumulation by isolated mitochondria was independent of phosphorylation, since neither 2,4-dinitrophenol nor methylene blue had appreciable effect on potassium accumulation, but inhibited phosphorylation completely. Moreover, ascorbate, when used as a substrate, gave the same rate of potassium accumulation as did *a*-ketoglutarate or succinate, although no phosphorylation occurred as a result of ascorbate oxidation. The oxidation of ascorbate was mediated by cytochrome oxidase, as judged, from the requirement for added cytochrome c and from the insensitivity to diethyldithiocarbamate.

Mitochondria, when isolated, show a rapid loss of lipide phosphate during incubation. Since isolated mitochondria tend to lose their accumulated potassium after about 40 minutes of incubation, it was deemed possible that the loss of phospholipides destroys the integrity of the diffusion-limiting barrier, allowing the leaching out of the accumulated ions.

The breakdown of phospholipides could be partially reversed by the addition of glycerophosphate, acetylcholine and CMP. The results indicated a synthesis of lecithin. Studies with P^{32} labelled inorganic phosphate indicated that the phosphate incorporated into the lecithin came preferentially from an internal pool of phosphate in the mitochondria.

The synthesis of lecithin had no effect on the gradual decrease of the rate of respiration during incubation, but reversed completely the usual decrease in the rate of phosphorylation. The synthesis of lecithin maintained the rate of potassium accumulation only to the extent to which it decreased the rate of total phospholipide loss. It is assumed that the lecithin prevents the back diffusion of accumulated ions. Judging from experiments in which the synthesis of lecithin occurred prior to the measurement of respiration, phosphorylation and accumulation, all the effects are due to the lecithin per se and not due to the actual process of synthesis.

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THE ASEPTIC CULTURE OF ISOLATED BUDS OF PSEUDOTSUGA TAXIFOLIA [1, 2]

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The sterile culture of isolated plant tissues and organs has been an effective technique in studying the physiology of plant development. Although extensive work has been reported on angiosperm tissues (8), few comparable studies with gymnosperm tissues have been made. Ball (1) described a medium for the successful culture of callus tissue from *Sequoia sempervirens,* and Loewenberg and Skoog (10) for callus tissues from 2 species of Pinus. Reinert (15) and Reinert and White (16) described methods for successful culture of normal and tumorous tissues of *Picea glauca.* Several studies involving use of aseptic culture of gymnosperm embryos have also been reported (3, 4, 6, 7, 9, 11, 18, 21, 22). No reports are known concerning the culture of isolated dormant buds of gymnosperms. The present paper describes a method for the culture in vitro of isolated dormant buds of Douglas fir, *Pseudotsuga taxifolia* and reports certain effects of components of the nutrient medium on the development of the buds in culture.

MATERIALS AND METHODS

In Berkeley, the leaves within the terminal bud of *Pseudotsuga taxifolia* begin their differentiation as primordia in April and May. By late August the development of leaf primordia is complete within the bud which then enters a period of rest. During the winter rest period, extending from about August to February, the terminal bud remains in a relatively inactive state. The bud is completely formed; it is dark brown in appearance due to the tightly enclosing bud scales within which is the axis bearing all the leaf primordia for the next season's vegetative growth (fig 1). Between February and April the dormant bud expands to become the fully developed shoot. In February the buds swell during the development of the leaf primordia to almost mature leaves. Then the outer brown scales loosen, the inner transparent scales elongate and are pushed out, and finally in early April the full-sized bud opens and the leaves emerge. Within 3 to 4 weeks the leaves and the shoot reach their maximum size. Already at the shoot apex the meristematic activity, which leads to the formation of the new terminal bud, has begun the laying down of new leaves.

In the present study dormant terminal buds of *P. taxifolia* were collected from a single old tree in Berkeley throughout the periods of October to January of 1955 to 1956 and 1956 to 1957. The buds, together with a very short part (1 cm) of the branch at their base, were excised from the tree. The branchlets were first cleaned by a succession of thorough washes in tap water, detergent solution ("Labtone"), tap water and distilled water. The branchlets were then surface sterilized by shaking in a 2.5 % solution of Sanichlor (sodium hypochlorite 5.25 % by weight) for 3 to 5 minutes and were finally rinsed in sterilized double distilled water. The bud scales were removed aseptically with forceps and the naked buds (fig 2) were then excised at the base and placed on the surface of the agar medium in 10 cm Petri dishes.

It is to be noted that during the winter rest period slight bud enlargement occurs (fig 2). In all of the experiments reported below, buds collected in the month of November were used. Two groups of buds, 1 collected in early November and 1 in late November, gave almost identical results. Preliminary experiments indicated that the culture requirements of buds collected earlier in the year differ somewhat from those reported here in being more complex. A detailed analysis of the dormancy of the buds and its physiological control by internal and external conditions was not made as a part of this study.

Six buds were placed in each dish containing 25 ml of the medium. Transfers were made every 3 to 4 weeks. At the time of the 1st transfer, the buds were moved into 90 × 50 mm crystallizing dishes containing 35 ml of medium. The buds were transferred in a transfer room previously sterilized by ultraviolet irradiation; dissection instruments were sterilized by immersion in 95 % ethyl alcohol and subsequent flaming. Contaminations were rare.

The buds were grown in a covered greenhouse which permitted only diffuse daylight from the sides. The temperature was not constant, varying from 27° C to 35° C over the period of the experiments. In addition to diffuse daylight, supplemental illumination was provided 12 hours each day by 18 30 watt Warmtone fluorescent tubes (Sylvania F 30 T 8/ W.W.), giving an average illumination of 550 ft-c at the Petri dish surface.

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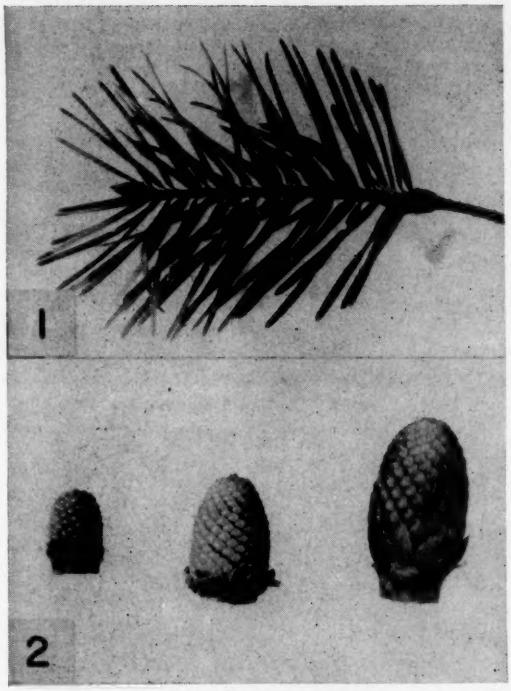


FIG. 1. One year old branch of *Pseudotsuga taxifolia* showing the winter resting bud and the leaf arrangement along the axis. X 1.

FIG. 2. Isolated buds of *Pseudotsuga taxifolia* after being descaled, and prior to culturing on nutrient medium. (*left to right*) Buds collected in October, December. and January. X 6.

The basic medium used in these studies consisted of the usual macronutrient salts in proportions based on a medium devised by Dr. R. B. Walker (University of Washington, Seattle) from water culture experiments on seedlings of *P. taxifolia* (table I). In addition, salts of micronutrient elements and the organic supplements glycine, thiamin, nicotinic acid, pyridoxine and L-tyrosine were used. After a series of trials, to be described, sucrose was selected as the carbohydrate source and routinely added at 2 %. Powdered Bacto agar (Difco) was added to the medium at 0.4 %. Pyrex redistilled water was used for all media. Unless noted otherwise, all components of the media were mixed together and steam autoclaved at 15 lbs/sq in for 20 minutes. Certain of the organic supplements tested were filtered cold with a Seitz filter and added to the autoclaved medium before pouring into the dishes. All glassware was thoroughly washed in tap water, soaked overnight in detergent solution, rinsed in tap water and distilled water, air dried, then sterilized by dry heat.

Initial studies using a liquid medium were generally unsuccessful, since the buds sooner or later sank below the surface and subsequently failed to develop. With the soft agar medium, development was also stopped if the buds were submerged. Thus oxygen appears to be essential to bud development at this stage.

A number of growth factors and organic supplements were added separately and in various combinations to the basic medium in order to study their effect on the expansion of leaves and the development of the buds into elongate shoots. In no case was completely normal shoot development achieved in vitro, although under certain conditions apparently normal expansion of certain leaves and limited bud development were achieved. In each experiment many buds were used, varying from 6 to 10 buds for each test. In the figures, a single bud representative of the particular treatment has been photographed. Figures 3 to 24 are at the same magnification so that direct comparison of the various treatments may be made.

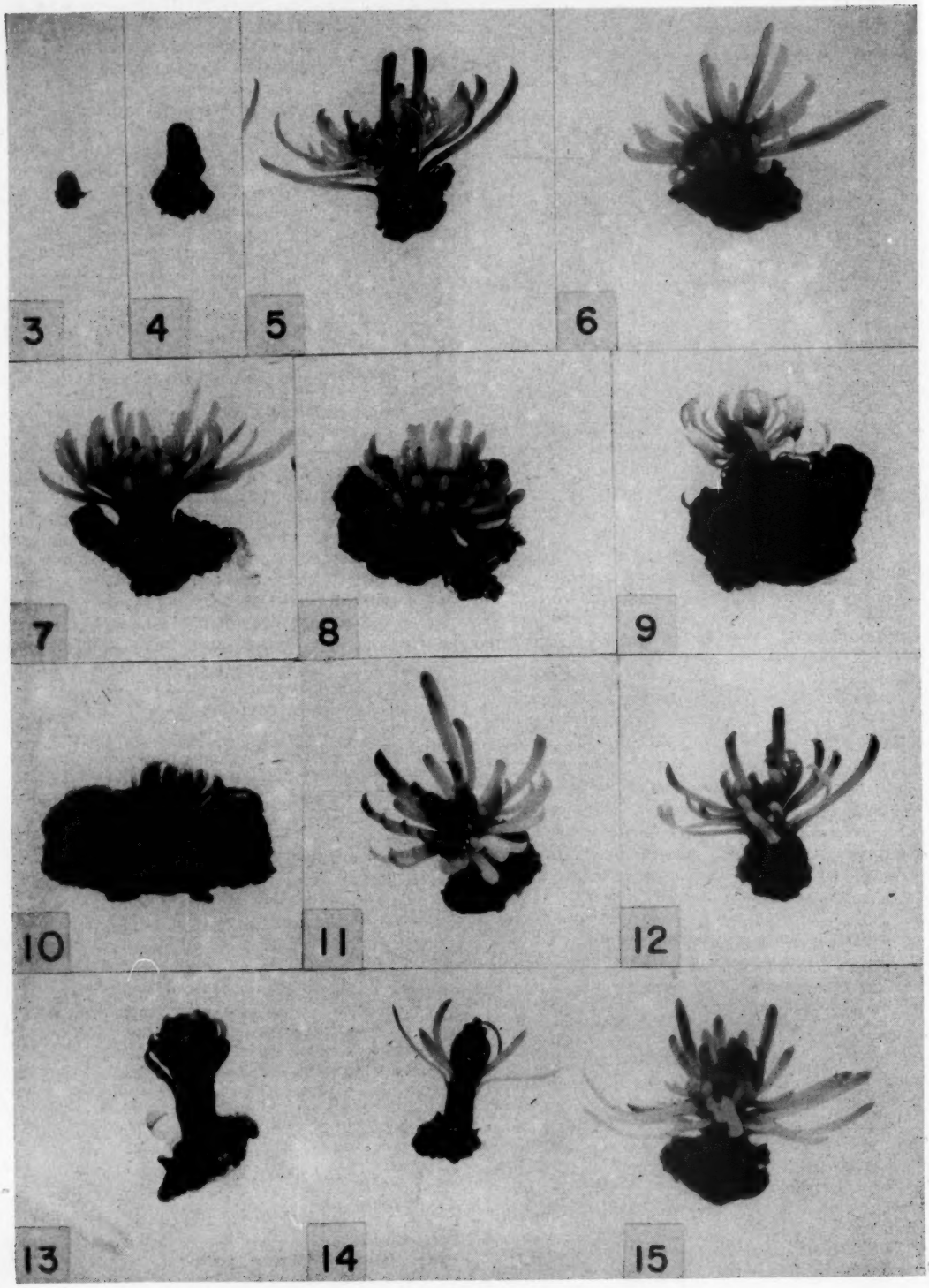
RESULTS

EXPERIMENTS WITH SUGAR: Although excised buds of *P. taxifolia* are green, contain chlorophyll and require light for their development in vitro, they fail to grow on a sugar-free medium (cf. figs 3, 4, 5). Sucrose, glucose, fructose and a mixture of glucose and fructose were tested in concentrations from 1 to 6 %, either added to the medium separately or autoclaved with the other constituents of the medium. The buds clearly require a carbon and energy source in the form of a simple sugar and develop almost equally well on sucrose (1 to 4 %), fructose (1 to

TABLE I

COMPOSITION OF BASIC MEDIUM USED FOR CULTIVATION OF ISOLATED VEGETATIVE BUDS OF PSEUDOTSUGA TAXIFOLIA

COMPONENTS	MG/L
Macronutrient Salts	
$Ca(NO_3)_2 \cdot 4H_2O$	236
KH_2PO_4	86
KCl	149
$MgCl \cdot 6H_2O$	71
$MgSO_4 \cdot 7H_2O$	37
$NH_4H_2PO_4$	57
Micronutrient Salts	
$FeCl_3$	1.5
H_3BO_3	1.5
$MnSO_4 \cdot H_2O$	4.5
$Na_2MoO_4 \cdot 2H_2O$	0.2
$ZnSO_4 \cdot 7H_2O$	1.5
KI	0.075
Organic Supplements	
Glycine	3.0
Nicotinic acid	0.5
Thiamin HCl	0.1
Pyridoxine HCl	0.1
L-Tyrosine	7.25
Sucrose	20 gm/l (2 %)
Difco Bacto-agar	4 gm/l (0.4 %)
Pyrex glass double distilled water	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3 %), glucose (1 to 4 %) or mixtures of glucose and fructose (each at 1 to 3 %). The best growth occurred in sugar concentrations of 1 to 2 % and all the sugars tested seemed to support growth almost equally well. The typical appearance of an excised bud cultured for 2 months on 2 % sucrose in the basic medium is shown in figure 5. The absorbing base of the bud in contact with the medium has expanded as a callus tissue; a number of leaves have expanded remarkably as compared with their size on excision (fig 3) although the stem axis shows relatively little elongation. Sucrose at 2 % was arbitrarily chosen as the sugar for the basic medium. It is known (2) that sucrose when autoclaved with other nutrient medium constituents produces small amounts of both glucose and fructose. Excised buds grown on the basic medium containing sugars do not develop in the dark, so that the light requirement is more complex than a requirement for photosynthesis.

The development of the isolated buds on the basic medium containing 2 % sugar, although remarkably good for an isolated organ grown in vitro, is far from that observed in the intact branch of a tree. In an attempt to achieve more complete development in vitro, a wide variety of substances was tested by their addition to the basic medium containing 2 % sucrose.

Experiments with Auxins and Related Compounds: The first successful attempt at maintaining coniferous tissue in culture was made on *Sequoia sempervirens* by Ball (1), who incorporated indole-3-acetic acid (IAA) in the medium at 10^{-6} g/l. Loewenberg and Skoog (10) were able to maintain a callus tissue of Pinus on a mineral medium containing sucrose, malt extract and the auxin, *a*-naphthalene acetic acid (NAA) at 10^{-6} g/l. Reinert and White (16) found that 2,4-dichlorophenoxyacetic acid (2,4-D) was more effective than IAA in stimulating tissue growth of *Picea glauca* grown in vitro.

In the present investigation 5 well-known auxins, IAA, NAA, 2,4-D, indolebutyric acid (IBA) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2 reputed antiauxins, 2,6-dichlorophenoxyacetic acid (2,6-D) and 2,3,5-triiodobenzoic acid (TIBA) were tested at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M for their effects on the growth of the cultured vegetative buds of *P. taxifolia* (figs 6-12).

Some leaf expansion, as well as callus tissue formation, occurred in the absence of auxin in the medium, suggesting that the buds may be auxin autotrophs. The addition of auxin to the medium stimulated callus tissue development and tended to inhibit leaf expansion. With increasing auxin concentrations, the inhibitory effect on leaf development became more evident.

Figures 6 to 12 illustrate the effects of these growth substances at a concentration of 10^{-6} M. Particularly evident is the effect on callus tissue formation, which is especially marked in treatment with MCPA, 2,4-D and NAA. Here the extensive growth of the basal callus tissue has occurred at the expense of leaf development. At higher auxin concentration leaf development was generally retarded and at 10^{-4} M, death of the bud usually ensued.

The occurrence of browning of the basal callus tissue was noted early during the culture work. The callus tissue was initially healthy and golden brown in color, but later became dark brown. This pigmentation was not prevented by adding L-tyrosine to the medium as described by Reinert (15) nor were any attempts to eliminate ultimate browning of the tissue successful. Some attempts were made to cultivate the callus tissue itself in culture but the inevitable browning of the tissue ultimately led to the cessation of tissue growth and loss of the culture.

TIBA and 2,6-D at 10^{-6} M had little effect on bud development; at 10^{-5} M they caused retarded leaf expansion and at 10^{-4} M, no callus tissue developed. At all concentrations of 2,6-D bud leaves were of a distinctive deep green color.

In general, it appears that if the excised buds require auxin for their normal development, some endogenous auxin is already present in the tissues and externally supplied auxin has no further stimulatory effect on bud development. At higher concentrations

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Fig. 3. Isolated bud collected in November, at the beginning of the culture period. X 3.

Figs. 4-15. Isolated buds cultured on different synthetic nutrient media for 2 months. All cultured buds were grown on the basic medium with the additions noted for each figure. All at the same magnification. X 3.

Fig. 4. Basic medium lacking sugar and containing no other energy or carbon source.

Fig. 5. Basic medium including 2 % sucrose (cf. table I).

Fig. 6. Indoleacetic acid at 10^{-6} M.

Fig. 7. Indolebutyric acid at 10^{-6} M.

Fig. 8. *a*-naphthaleneacetic acid at 10^{-6} M.

Fig. 9. 2,4-dichlorophenoxyacetic acid at 10^{-6} M.

Fig. 10. 2-methyl-4-chlorophenoxyacetic acid at 10^{-6} M.

Fig. 11. 2,6-dichlorophenoxyacetic acid at 10^{-6} M.

Fig. 12. 2,3,5-triiodobenzoic acid at 10^{-6} M.

Fig. 13. Gibberellic acid at 10 mg/l.

Fig. 14. Gibberellic acid at 0.1 mg/l.

Fig. 15. Basic medium lacking gibberellic acid (control).

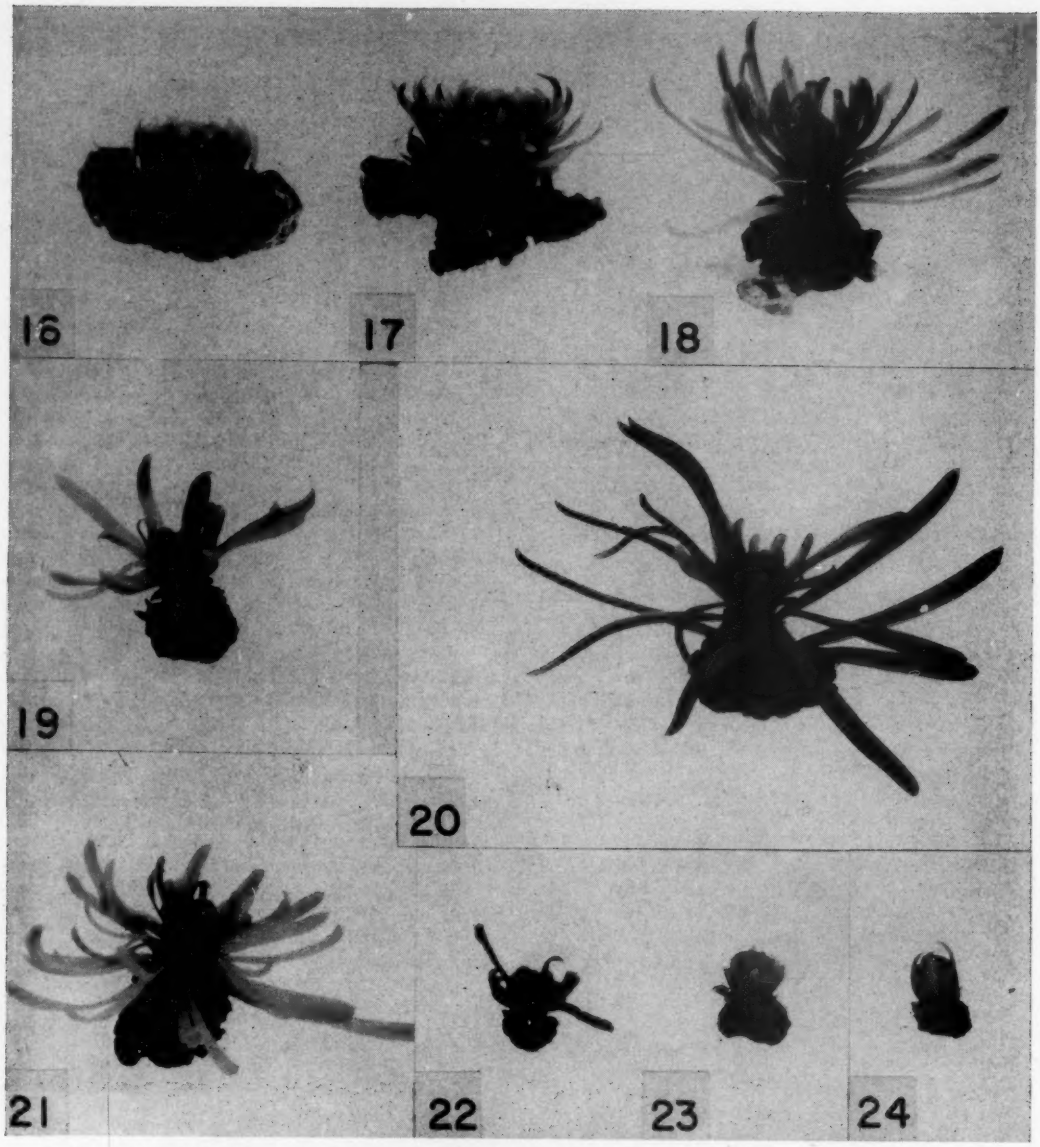

FIG. 16-24. Isolated buds of *Pseudotsuga taxifolia* cultured on different synthetic nutrient media for 2 months. Additions were made to basic medium as noted. All at the same magnification. X 3.

FIG. 16. Kinetin at 1 mg/l.
FIG. 17. Kinetin at 0.01 mg/l.
FIG. 18. Kinetin at 0.0001 mg/l.
FIG. 19. Adenine sulfate at 20 mg/l.
FIG. 20. Urea at 5×10^{-4} M.
FIG. 21. Thiourea at 1.3×10^{-6} M.
FIG. 22. Casein hydrolysate at 0.3 %.
FIG. 23. Coconut milk at 5 %.
FIG. 24. Yeast extract at 0.01 %.

the externally supplied auxins become inhibitory to leaf expansion and upset the balance in development so that callus tissue formation becomes predominant. Toxic levels are reached at concentrations of 10^{-4} M.

EXPERIMENTS WITH OTHER GROWTH REGULATING SUBSTANCES: The effects of gibberellic acid on intact plants, excised plant parts and on tissues in culture, have been reported (see review by Stowe and Yamaki (23)). In work on gymnosperms, Marth, Audia and Mitchell (12) reported that pine seedlings showed relatively little response to gibberellic acid treatment. In the present study, gibberellic acid was tested in a medium lacking auxin at concentrations of 0.1 and 10 mg/l. The solution was sterilized by Seitz filtration before addition to the medium. In figures 13 to 15 the characteristic effects of gibberellic acid are illustrated. Leaf expansion was restricted but the main bud axis typically showed elongation. This elongation response was not noted in any other medium tested. A second important effect of gibberellic acid at the concentrations tested was the invariable death of the bud, which began by blackening of the apical area and then progressive blackening of the entire bud leading to its death.

The cell division factor of Miller et al (13), 6-furfurylaminopurine or kinetin, was also tested for its effect on bud development over a concentration range from 0.0001 to 1.0 mg/l. In their studies on tobacco stem pith tissues, Skoog and Miller (19) have shown the stimulatory effect of kinetin in the presence of IAA on cellular proliferation of the callus tissues and, under appropriate concentrations of these interacting factors, on bud initiation. The effect of kinetin on the development of isolated buds of *P. taxifolia* is illustrated in figures 16 to 18. In general one can say that the higher the concentration of kinetin, the greater is the growth of the callus tissue at the base of the bud, and at the same time, the less the expansion of the leaves. In the presence of 1.0 mg/l bud expansion is inhibited while the callus grows well although with time it undergoes the usual browning. At the lowest concentration tested, the buds showed slightly better development than the controls without kinetin. Thus, increasing concentrations of kinetin favor callus proliferation at the expense of normal leaf expansion.

It was interesting to test the effect of the closely related substance, adenine, which has been repeatedly suggested as a leaf-growth factor (Bonner and Haagen-Smit (5); Went (24)) as well as one of the factors involved in bud initiation (Skoog and Tsui (20)). Adenine sulfate was added to the medium in concentrations ranging from 20 to 60 mg/l in the presence of 10^{-6} M IAA. At all concentrations tested, adenine sulfate caused reduced bud development and leaf expansion, although the lowest concentration tested, 20 mg/l, was least inhibitory (fig 19). Extensive experiments to test the interaction of IAA and adenine were not made.

Because of studies showing the effective utilization of urea as a nitrogen source by certain tissues in culture (Riker and Gutsche (17), Nickell and Burkholder (14)), a series of experiments was set up to test the utilization of urea by the excised buds. Solutions were sterilized by Seitz filtration and added to the medium to give final concentrations of 10^{-1}, 10^{-2} and 5×10^{-4} M urea. No IAA was present in the medium. At the 2 higher concentrations, blackening of the buds occurred within a few days, followed by death of the buds. At the lowest concentration, however, bud development was excellent and leaf expansion the best that was achieved in any medium (fig 20). The leaves were characteristically elongate and the lamina flat and wide. Callus tissue at the base of the bud developed only to the extent found in the control medium. Thiourea at somewhat lower concentrations (1.3×10^{-4}, 1.3×10^{-5} M) inhibits bud expansion while at still lower concentrations (1.3×10^{-6} M) it has little or no inhibitory effect (fig 21), and apparently serves as a nitrogen source.

EXPERIMENTS WITH COMPLEX ORGANIC SUPPLEMENTS: In further attempts to achieve complete expansion of isolated buds in vitro, various mixtures containing organic materials were tested as components of the medium. Coconut milk, yeast extract, casein hydrolysate and watermelon juice were tested at a number of concentrations. These experiments are summarized in table II together with the results.

TABLE II

EFFECT OF COMPLEX ORGANIC SUPPLEMENTS ON BUD DEVELOPMENT

ORGANIC SUPPLEMENT *	CONCENTRATION %	AUXIN ADDED	RESPONSE OF BUDS
Coconut milk	5, 10, 20	none	Inhibition of bud expansion
Yeast extract	0.01, 0.1, 0.5	IAA absent and 10^{-6} M IAA	Inhibition of bud expansion
Casein hydrolysate	0.3, 1.0, 10.0	none	Slight growth at lower concentrations, inhibition at highest concentration
Watermelon juice	0.1, 1.0, 10.0	none	Good growth at 0.1 %, little at 1 %, inhibition at 10 %

* All sterilized by cold Seitz filtration except yeast extract.

Characteristic responses to these mixtures are illustrated in figures 22 to 24. All mixtures caused inhibition of bud development at most concentrations tested. Only watermelon juice at the lowest concentration was noninhibitory. Since these mixtures were tested over concentration ranges generally reported to be stimulatory to either bud or callus tissue growth in a variety of systems, it is apparent that the isolated buds of *P. taxifolia* are relatively sensitive to the added components of the medium. It is suggested that a substance or group of substances common to all these mixtures inhibits bud development. No attempt to identify the inhibitory substances was made.

Discussion

It was the initial purpose of these studies to achieve maintenance of isolated leaves of *P. taxifolia* in vitro so that certain aspects of cellular differentiation in leaves could be studied. All attempts to maintain isolated leaves of this plant in culture, whether isolated from the immature bud or taken from the fully expanded branch ended in failure. Attention then turned to culture of the whole bud, taken from the tree at a dormant immature stage and cultivated in vitro. Successful culture of these buds has allowed the study of cellular differentiation of the leaves isolated from the influence of the whole tree and thus the object of the research was achieved. A report of sclereid differentiation in leaves of Pseudotsuga will be published elsewhere.

The successful maintenance in vitro of isolated buds of *P. taxifolia* through a considerable part of their early development opens up a new and useful approach to a number of fundamental problems in the development of gymnosperms. Clearly, further work remains to be done before completely normal development is achieved in vitro.

A number of interesting facts have been established by the present studies. Mature buds of *P. taxifolia* may be taken from the tree in the middle of the winter rest period, isolated as descaled buds and stimulated to develop immediately by culture in a nutrient medium. Such naked buds require available oxygen, light for processes other than photosynthesis alone, a carbon and energy source, preferably a sugar such as sucrose, and the mineral elements of a nutrient culture solution.

Under these conditions, leaf expansion, especially of the first formed basal leaves, will proceed to almost normal size. Elongation of the main axis is much reduced as compared to development on the intact plant, and younger leaves in the bud fail to expand. Preliminary experiments on the effects of cold treatment of these isolated buds indicated that some beneficial effect might result from cold temperatures.

The isolated buds apparently are not limited in their development by an auxin requirement; on the contrary, evidence suggests the buds may be auxin-autotrophs. The addition of auxins to the medium almost always led to reduced leaf expansion. Other studies not reported here show that auxin altered also the course of cellular differentiation in the leaves of the buds. Auxin added to the medium appeared to stimulate unorganized cellular proliferation at the base of the buds leading to callus tissue formation, usually at the expense of leaf and bud development. Callus development was accentuated by addition of kinetin. In no case was there any initiation of root primordia.

It was not the intention of the present work to complete a detailed nutritional study of the isolated buds. Thus many important aspects of the nutrition of the buds remain unexplored. Further studies in these unexplored directions may make possible the development of isolated buds into normal elongate shoots in vitro.

Summary

The aseptic culture of isolated vegetative buds of *Pseudotsuga taxifolia* has been studied. A technique was developed for sterilizing the bud taken from the tree in the middle of the winter rest period and for culturing it on an agar nutrient medium. It was shown that such isolated buds require available oxygen, a carbon and energy source such as one of the sugars, sucrose, glucose or fructose, and the mineral elements of the nutrient medium. Buds fail to develop in the absence of light but develop well in a nutrient medium if grown under white fluorescent light. Added auxins at 10^{-6} M had no stimulatory effect on bud or leaf expansion; higher concentrations were inhibitory to bud development and stimulated callus tissue proliferation at the base of the bud. Gibberellic acid, although stimulating axial elongation of the bud, always caused death of the bud at concentrations tested. Of a number of organic substances tested, only urea had a beneficial effect on bud development. Other compounds and organic supplements, including coconut milk, yeast extract, casein hydrolysate and watermelon juice, were inhibitory to bud expansion. On the best medium developed, isolated buds were maintained for over 2 months in vitro during which they developed from tiny naked buds to large, partially expanded shoot systems bearing mature fully expanded basal leaves.

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AN IMPROVED TECHNIQUE FOR THE GROWTH OF CHLORELLA IN $C^{14}O_2$ [1]

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Previous attempts by the author to produce uniformly labeled C^{14} compounds of high specific activity by growing algae photosynthetically in the presence of $C^{14}O_2$ have been unsatisfactory because of frequent failure of the algae to grow. The method originally employed (5) was a modification of that described by Roberts et al (9). Failure of growth was not due to cultural inadequacies or secondary infection but appeared to be associated with a high isotopic ratio, and the initially high concentration of CO_2. It was felt that the latter difficulty would be overcome by the use of a device permitting fractional addition of CO_2 during growth, and that the most efficient labeling would be achieved by recirculation of the gas. Although an apparatus utilizing the principle of recirculation has been described (3, 4), the method involves the generation of the total CO_2 at one time. The present paper describes a simple apparatus for the growth of algae in the presence of continuously circulating $C^{14}O_2$, added fractionally throughout the growth period.

MATERIALS AND METHODS

PREPARATION OF INOCULUM: A 500 ml flask containing about 300 ml of medium (table I) is inoculated with a loopful of *Chlorella pyrenoidosa* (No. 7516, American Type Culture Collection). A gas mixture consisting of 5 % CO_2 in 95 % N_2 or in air is bubbled through the medium. Illumination is provided by a 75 watt bulb placed about 15 cm from the growth vessel. Aseptic technique is not required since the absence of an organic carbon source renders contamination unlikely. After incubation at room temperature for 5 to 7 days, an optical density-dry weight curve is determined by reading variously diluted samples of the culture at 540 mμ (Spectronic 20) and drying them to constant weight.

APPARATUS: The apparatus, as schematically shown in figure 1, consists of a gas generator assembly

[1] Received March 23, 1959.

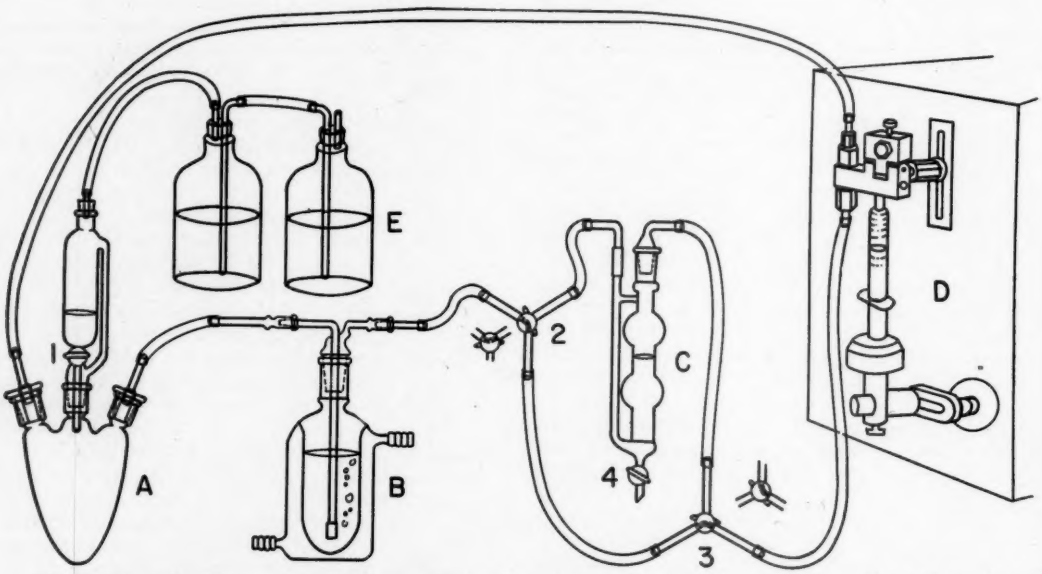

FIG 1. Schematic diagram of growth apparatus. Components of the gas generator assembly (A) consist of items No. 93950, No. 94750, No. 8825 and No. 6680 obtainable from the Corning Glass Works, Corning, New York. Growth flask (B) is assembled from No. 96320, No. 98075, No. 6680 and No. 6640, also available from Corning. Absorption tower (C) consists of No. 9852 obtainable from Microchemical Specialties Co., Berkeley, California. The pump (D) was a Filamatic Model A, National Instrument Co., Baltimore, fitted with a 10 ml syringe lubricated with silicone stopcock grease. The two 3-way stopcocks are obtainable from Corning as item No. 7450.

(A), a jacketed growth flask (B), an absorption tower (C), an automatic pipetting machine (D) serving as a pump, and a pressure equalizing assembly (E). These parts are connected by Tygon tubing and by two 3-way stopcocks as indicated. The stopcocks and all standard taper joints are lubricated with silicone grease.

OPERATION: Barium carbonate-C^{14} is diluted with carrier to the desired specific activity (3 to 5 mC/mM) and added to the generator flask (A) in an amount sufficient to generate CO_2 equivalent to 5 % of the total volume of the apparatus. With stopcock 1 closed, an excess of 40 % perchloric acid is placed in the funnel above the generator flask. The growth flask (B) contains 150 ml of medium with 0.1 mg of algae as determined by optical density and is cooled by running water to about 24° C. Illumination is provided by a 100 watt incandescent lamp placed 2.5 cm from the growth flask. The absorption tower (C) is filled with about 60 ml of 0.1 *N* NaOH, and each of the pressure equalizing bottles filled with about 900 ml of 0.1 *N* HCl as shown. Stopcocks 2 and 3 are set as indicated in figure 1. The pump is started and a small amount of acid is run on to the barium carbonate by opening stopcock 1. Additional amounts of gas are generated at 1 to 2 day intervals by adding additional acid to flask (A). The gas is circulated at 150 to 170 ml per minute. After 7 to 10 days, when full growth is obtained, the pump is turned off, stopcocks 2 and 3 set in the alternate positions shown, and the pump started again thus circulating the gas through the absorption tower (C). About 6 hours later when absorption is completed, the pump is stopped and the alkali from the absorption tower is dropped into 0.1 M $BaCl_2$ in 0.1 M NH_4Cl by opening stopcock 4. The resulting precipitate of $BaCO_3$ is washed with hot water until free of chloride and dried with alcohol.

FRACTIONATION OF ALGAE: The particular procedure used will, of course, depend upon the nature of the components to be isolated. The fractionation method used in this laboratory is based upon our interest in obtaining labeled amino acids and nucleic acids.

The grown algae are centrifuged and washed with ice-cold distilled water. The supernatant fluids are pooled and HCl is added to bring the pH to about 2.0.

TABLE I

COMPOSITION OF CHLORELLA MEDIUM

KNO_3	2.0	g
KH_2PO_4	1.0	g
Na_2HPO_4	0.245	g
$MgSO_4 \cdot 7H_2O$	0.500	g
$FeCl_3$ solution (0.1 %)	1.0	ml
Micronutrient solution*	0.1	ml
Distilled water	1000	ml
pH 6.2		

* Composition given in (7).

The CO_2 evolved is trapped in alkali and converted to $BaCO_3$ as described above. The pooled supernatant solutions constitute the "supernatant medium" fraction. The sedimented algae are washed with alcohol and ether and the washings are pooled. This pool represents the "alcohol-ether" fraction and contains lipids, lipoproteins, chlorophyll, carotenoids, etc. The sediment from the ether extraction is dried and is then extracted for about 8 hours with 10 % NaCl in a boiling water bath with constant stirring. After cooling and centrifugation, the supernatant fluid is collected and constitutes the "NaCl supernatant" fraction. The sediment is washed with alcohol and ether, and dried (NaCl sediment fraction) and the washings are added to the alcohol-ether fraction. The NaCl supernatant fraction is adjusted to pH 4.0 to 4.5 with acetic acid, and 3 volumes of cold ethanol are added. The precipitate which forms after standing overnight at 5° C consists largely of mixed nucleic acids and is washed with alcohol and ether, and then dried. The residual supernatant solution contains metabolic intermediates and amines. The NaCl sediment contains mainly protein and is hydrolyzed in a sealed tube, either with 6 *N* HCl-formic acid (1:1) for 15 hours or with 14 % $Ba(OH)_2$ for 18 hours at 121° C. After removal of the acid in vacuo or of the $Ba(OH)_2$ with solid CO_2, the hydrolysate is resolved into individual amino acids on columns of Amberlite IR-120 (8).

ASSAY: Samples to be counted were suitably diluted and evaporated to infinite thinness on metal planchetts. Counting was done in the Geiger region with an Atomic Scaler, model 1091, equipped with a Packard gas flow counter. All counts were made with a 95 % probability of 4 % error or less. Typical results for 2 runs are given in table II.

TABLE II

DISTRIBUTION OF ACTIVITY IN ALGAE FRACTIONS

	COUNTS PER MINUTE × 10^8			
	RUN NO. 1		RUN NO. 2	
		%		%
Total counts added	133.2*		86.6*	
Recovered CO_2	1.7	1.3	8.1	9.3
Supernatant medium	2.7	2.1	1.6	1.9
Alcohol-ether fraction	34.7	26.1	20.7	23.9
NaCl supernatant fraction	74.0	55.6	44.1	50.9
NaCl sediment	9.0	6.8	5.3	6.1
Total counts recovered	122.1	91.9	79.8	92.1
Dry wt algae recovered (mg)	105		96.6	
C^{14} uptake by algae		88.4		82.1
Activity of algae (μC/mg)	126		81	

* These 2 figures are theoretical since the $BaCO_3$-C^{14} was not actually counted. The figures are based on 22.2 × 10^8 (d/min/mC) × 0.39 (counter efficiency) × 15 or 10 mC respectively.

Discussion

The failure of algae to grow in a medium containing large amounts of $C^{14}O_2$ may be caused primarily by 2 factors:

1. The initial presence of a large amount of CO_2 in the growth flask results in increased acidity of the medium to a level inhibitory to growth because the small inoculum initially present cannot metabolize the CO_2 fast enough to prevent its accumulation. The obvious remedy is to add CO_2 approximately as required, thus avoiding an initial excess.

2. Inhibition of growth may also be due to isotope effects resulting from radiation or isotope discrimination. Although C^{14} is a pure, low energy beta emitter, growth inhibition by radiation is difficult to rule out since beta particles may be produced and absorbed within the interior of the cell. M. Calvin (Personal communication, 1956.) has the impression that algae are relatively insensitive to radiation, though not particularly out of line with respect to other micro-organisms. Isotope discrimination may involve preferential reaction due to differences in the mass or in the stereo-configuration of the isotope (1). More specifically, Weigl and Calvin (10) have shown that algae exhibit a marked preference for C^{12} over C^{13} compounds and more so for C^{12} over C^{14} compounds. Furthermore, Buchanan et al (2) demonstrated that C^{14} labeled algae will preferentially exchange the C^{14} for C^{12}.

Earlier unpublished attempts by the author to grow algae using $BaCO_3$-C^{14} with isotopic ratios of 19 to 26 % repeatedly failed to yield growth, whereas isotopic ratios of 5 to 10 % reproducibly permitted growth. The possibility that cultural inadequacies or secondary infection were responsible for the failure of growth in the flasks containing high isotopic ratios was experimentally ruled out.

It is suggested that algae are capable of metabolizing only a limited percent of C^{14}, and when the limiting ratio of C^{14} to C^{12} is exceeded, growth is not possible. The solution of this problem appears to be avoidance of biosynthetic experiments in excess of this critical ratio which seems to be of the order of 10 %.

With use of the method described above it has been possible to produce C^{14} labeled algae of considerably higher activity than is commercially available. Consequently, labeled amino acids, purines, pyrimidines and other compounds of biological interest (7) can be isolated from such algae with considerably greater specific activity, permitting higher dilution with carrier compounds. Furthermore, the great increase in the mass of the algae during growth (approximately a thousand-fold) assures a high probability for random distribution of the C^{14} label.

Summary

An apparatus for the photosynthetic growth of algae in an atmosphere of $C^{14}O_2$ has been described. The 2 factors involved in the production of high activity algae appear to be the avoidance of a high initial concentration of CO_2, and the use of an isotopic ratio of about 10 % or less. Using the procedure and apparatus described, C^{14} labeled algae with an activity of the order of 100 μC/mg and with a high probability of random labeling have been prepared.

The author gratefully acknowledges the technical assistance of Miss Grace Ullman.

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THE EFFECTS OF TRIIODOBENZOIC ACID AND UREA ON THE RESPONSE OF CHLOROTIC LEMON (CITRUS LIMONIA) TREES TO FOLIAR APPLICATION OF IRON COMPOUNDS [1]

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The control of chlorosis due to faulty iron nutrition in citrus trees in alkaline and calcareous soils still presents many problems in several areas used for citrus production. Unpublished work by Bar-Akiva in Israel has led to the general finding that soil applications or foliar sprays of iron chelate compounds have often proved less successful with citrus trees than with many other crops (1), (5). Foliar sprays of iron chelates may, at best, produce only irregular green spotting of still chlorotic leaves. Soil applications of the iron chelate of hydroxyethylethylenediaminetetraacetic acid may prove successful in some instances (4) but are costly. Triiodobenzoic acid (TIBA) was reported to promote the mobility of iron and calcium applied in foliar sprays to tomato plants and apple trees (3), while urea was found to enhance the response to iron applied to chlorotic foliage of banana and citrus trees (6) (and private communication by R. Gosen). This paper describes the effects of TIBA and of urea on the movement of iron applied to normal and chlorotic citrus trees using radio active Fe^{59} as ferric chloride.

MATERIALS AND METHODS

PLANT MATERIAL: Eureka lemon trees were grown from seed in sand cultures under greenhouse conditions using the methods already described (2). After about 2 months growth with a complete nutrient solution (2) some of the trees were deprived of iron by transferring them to an iron deficient nutrient supply. Symptoms of typical iron deficiency appeared in a few weeks and became progressively more severe during growth. Trees were treated 3 or 4 months after change of iron supply. Normal trees bore 12 to 16 expanded leaves and chlorotic trees bore 8 to 10 small leaves at the time of the experiments.

EFFECTS OF TRIIODOBENZOIC ACID (TIBA): Each of the following 5 treatments was given on June 5 to 3 trees in each of 2 pots. Both complete nutrient and iron-deficient trees were used for the experiments in case the mobility of applied iron showed any difference in relation to the condition of the trees: 1) 100 ppm TIBA foliar spray; 2) 200 ppm TIBA foliar sprav; 3) 300 ppm TIBA foliar spray; 4) 250 ml 0.025 % TIBA applied to the sand; 5) water control foliar spray.

Two thousandths percent "Texofor" F 8 (an isooctyl *o*-cresol ethylene oxide condensate) was included as a wetting agent in the spray treatments. The TIBA was applied as a foliar spray to ensure that foliage on all parts of the tree received the application without complicating effects of translocation and root uptake. The application of TIBA to the sand was given in case this should prove more effective than direct foliar treatment. The TIBA sprays were followed by an application of 0.1 ml ferric chloride solution containing 0.3 μc Fe^{59} per ml. This was spread with a brush on the surface of the first fully expanded terminal leaf.

Five days after this application the treated leaf, and older and younger leaves, and a part of the root system were harvested from two trees in each pot, and the radio activity measured with a scintillation counter. The third tree in each pot was harvested for radioautography tests on comparable leaves.

The activity of treated leaves ranged from 1,118 to 1,422 cpm for the various treatments described above. There was no detectable activity, however, either in leaves older than that given the iron or in the newly formed younger leaves or in the roots. The radioautographs also showed no activity in any leaves, except that to which the iron had been applied.

A separate trial was also made using a foliage spray of ferrous sulphate (0.5 %) after the TIBA treatment described above had been given. In this test, as already observed in field trials in Israel, chlorophyll production occurred only as localized green spots 5 days later in the leaves to which iron was applied and no other leaves produced fresh chlorophyll.

EFFECTS OF UREA: In a second series of experiments, urea was used in conjunction with TIBA pretreatment to test its effect on the penetration and mobility of foliar applied Fe^{59} present as $FeCl_3$. The procedure was similar to that used previously and the following treatments were given: 1) 2 % urea followed by Fe^{59} as $FeCl_3$; 2) 2 % urea and 300 ppm TIBA followed by Fe^{59} as $FeCl_3$; 3) water control followed by Fe^{59} as $FeCl_3$.

After five days, the iron-treated leaves and those adjacent to them were examined for radioactivity. As before, only the treated leaves showed detectable activity and no downward translocation of iron could be detected. It was observed, however, that where urea had been applied to the leaves, the green spots which appeared about five days later were more

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numerous and more large than usual, and were not so localized. This effect was apparently due to the amalgamation of smaller spots.

It was thought that urea might have had some effect upon the mobility of iron, at least within those leaves to which iron had been applied. A second experiment was carried out to investigate this point, and visible chlorophyll production was used to detect the effect of iron applied as a foliar spray of ferrous sulphate solution (0.75 % $FeSO_4 7H_2O$). The following treatments were given on August 8 to each of three trees: 1) 0.75 % ferrous sulphate spray; 2) 300 ppm TIBA + 0.75 % ferrous sulphate spray; 3) 2 % urea + 0.75 % ferrous sulphate spray; 4) as above but applied to one-half only, of a leaf while the other half was covered with polythene sheet.

The characteristic green spots appeared about seven days later in the treated leaves. Figure 1 shows the appearance of treated leaves photographed after an interval of 20 days. It is evident that urea greatly increased the response of treated leaves to foliar applied iron when assessed in terms of area and uniformity of chlorophyll production. Leaf No. 4 which was covered on the right side when sprayed, produced chlorophyll only in the sprayed half.

Discussion

The effect of urea in increasing the extent and uniformity of chlorophyll production in iron-sprayed chlorotic citrus leaves might be interpreted in terms of improved penetration of the applied iron. As the presence of urea did not permit a response in the unsprayed half of the leaf it appears that urea influences only the penetration of iron into the leaf and does not affect iron distribution either within the leaf or from one leaf to another. Presumably urea has some effect on the permeability to iron either of the waxy cuticle or perhaps of the cell membranes.

Fig. 1. The effect of urea upon the penetration of foliar applied iron in chlorotic lemon leaves as measured by the formation of chlorophyll. Leaf No. 1, 0.75 % $FeSO_4$; leaf No. 2, 0.75 % $FeSO_4$ 300 ppm TIBA; leaf No. 3, 0.75 % $FeSO_4$ 2 % urea; leaf No. 4, 0.75 % $FeSO_4$ 2 % urea, only the left half of the leaf was sprayed.

It appeared that even when the presence of urea had resulted in improved responses to foliar applied iron and presumably in better penetration, the combined use of TIBA and urea did not promote the translocation to other leaves of any iron which had penetrated the leaf to which it was applied. The effects of TIBA on iron translocation reported by Kessler and Moscicki (3) for tomato and apple foliage was not, therefore, confirmed for lemon leaves in our experiment.

From a practical standpoint the combined use of urea and ferrous sulphate in foliage sprays applied at intervals during the year, appears to be a promising economical approach to the problem of controlling citrus chlorosis due to iron deficiency in calcareous or other alkaline soils. It is likely that the spray coverage must be relatively complete in order to obtain satisfactory results.

Summary

The effects on utilization of foliar applied iron as radioactive $FeCl_3$ of the application of triiodobenzoic acid (TIBA) as a spray to foliage or to roots of chlorotic citrus trees was investigated. No beneficial effects either on penetration or mobility of the applied iron could be detected. The application of 2 % urea in conjunction with ferric chloride or ferrous sulphate resulted in more uniform chlorophyll production in iron-sprayed leaves. Urea did not however promote any movement of iron across the leaf or from one leaf to another. Combined use of urea and TIBA showed no improvement over the effects of urea alone. It is concluded that urea promotes the more uniform penetration of iron applied to the leaves.

One of us (A. Bar-Akiva) is grateful to the British Council for the tenure of an overseas scholarship and for a grant for the purchase of the radioactive iron. We thank Mr. C. P. Lloyd-Jones for measurement of radioactivity and Mr. D. Aliband for the photograph.

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EFFECT OF VERNALIZATION ON HEAT RESISTANCE IN TWO VARIETIES OF PEAS [1]

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Practically all studies of vernalization have been concerned with the induction of a reproductive system. In a few cases, however, it has been shown that a period of cold given during germination will affect the subsequent growth and developmental behavior of the plants, independent of the flowering response. In winter Petkus rye, Gregory and Purvis (2) have shown that there is a reduction in tiller number as a consequence of a cold treatment. Although it is difficult to separate the flowering effects from the vegetative ones in this grass, there are indications that the effect on vegetative development may in fact be independent of the flowering behavior. Highkin (3[3]) has shown that in peas vernalized in such a manner as to prevent the hastening of flowering, growth is suppressed as a result of that vernalization treatment.

The after effects of a cold treatment are also apparent in the response of oats to high temperature. Coffman (1) has shown that spring varieties are less heat resistant than winter varieties. His data also show that a cold treatment given prior to a high temperature treatment will increase the heat tolerance of the winter varieties.

Thus it appears that the changes occurring in the plant during vernalization, of which we know literally nothing, have a profound effect on the plant as a whole and are apparently independent of the effects on flowering behavior.

The present paper is concerned with the relationship between vernalization and heat resistance in two varieties of peas.

MATERIALS AND METHOD

Two varieties of peas were used in these studies: one a vernalizable, heat sensitive variety, Unica, the other a non-vernalizable, heat resistant variety L_5. The plants were grown in the controlled environmental conditions of the Earhart Laboratory of the California Institute of Technology. Seeds of both varieties were soaked in water until completely imbibed (4 to 5 hours). They were then planted in individual 9 ounce containers in a 50-50 mixture of vermiculite and crushed rock (based on volume). This mixture was watered with Hoagland's solution whenever necessary. The planted containers were stored in the dark at 4° C for 25 days. This period has already been shown (3[3]) to be of sufficient length to elicit maximum flowering and growth response in the vernalizable variety, Unica.

After 20 days the control seeds of both varieties were soaked in water and put to germinate at 23° C. In five days the control seeds had germinated to approximately the same degree as the cold-treated seeds had germinated in 25 days, i.e. they had just emerged.

After 25 days at 4° for the treated plants and 5 days at 23° for the controls both groups of plants were randomized and put into an artificially lighted chamber set at 26° C ± 1° C with a 16-hour photo period. The light intensity was approximately 1,000 to 1,200 ft-c. The number of leaves and stipules showing necrosis was taken as a measure of heat damage.

RESULTS

The results of these experiments are shown in table I. Vernalized plants were considerably less necrotic than controls in both varieties (columns 1 and 2 of table I).

The data presented in columns 3 and 4 show that the first node to flower in the heat susceptible variety

TABLE I.

EFFECT OF VERNALIZATION TREATMENT ON HEAT INJURY AND FLOWERING IN VERNALIZABLE AND NON-VERNALIZABLE STRAINS OF PEAS

VARIETY	HEAT INJURY*		1ST FLOWERING NODE**	
	EXP. 1	EXP. 2	26°	20/14
Unica				
Vernalized	6.5 ± 0.38	5.9 ± 0.44	19.0 ± 0.75	16.0 ± 0.57
Unvernalized	18.3 ± 1.01	16.1 ± 0.69	19.5 ± 1.0	18.6 ± 0.52
L_5				
Vernalized	8.2 ± 1.93	10.0 ± 0.46	9.6 ± 0.17	9.3 ± 0.52
Unvernalized	17.1 ± 0.77	19.9 ± 0.67	9.2 ± 0.16	9.1 ± 0.1

* Grown at 26° C. Figures given are mean number of necrotic leaves and stipules/plant ± standard error. Minimum number of plants/treatment = 18.

** Average 1st flowering node ± standard error. Minimum number of plants/treatment = 9.

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[2] This work was supported in part by the National Science Foundation (grants G-1385 and G-6153).

[3] All figures in the 1956 paper, reference (3), should have read "cm" instead of "mm".

Unica, was significantly lowered when grown at 20° C day temperature with a 14° C night following vernalization. The vernalization treatment was without effect when the treated plants were subsequently grown at 26° C due to devernalization at this high temperature (3[a]). The data also show that vernalization had no effect on the flowering of the heat resistant variety L_5 at either of the temperature environments.

Thus it seems that the physiological effects of an inductive cold treatment on flowering and on development of heat tolerance are independent.

The variety L_5 is classified as being heat resistant primarily because of its ability to complete a life cycle at high temperatures (as high as 30° C in artificial light) in a relatively short time—approximately four weeks. Nevertheless, heat resistance seems to break down with the onset of flowering, in that necrosis develops and the resulting seed are inviable. The physiological events leading to this inviability must have occurred prior to pod formation for high temperature grown plants which were removed to a normal temperature (20° C day, 14° C night) immediately following flower formation, failed to produce viable seed.

The breakdown of heat resistance in L_5 is prevented by vernalization just as in the case of the heat susceptible variety Unica, where vernalization reduces the high temperature damage and permits the development of viable seed. In the unvernalized controls of Unica, no seeds are formed at high temperature.

In both varieties, each plant that had been previously vernalized, formed at least one pod at the high temperature. There was an average of three viable seeds per plant. The size of the seeds was approximately one half that of seeds from plants grown at 20° C day, 14° C night.

Discussion

The experiments reported here extend those of Coffman (1), Gregory and Purvis (2) and Highkin (3[a]) in showing that vernalization has a much more profound effect on the plant than has been generally recognized. Vernalization leads to changes in the plant other than those associated with the process of reproduction. The present experiments show that at least one of these changes is a marked increase in heat resistance. That the induction of heat resistance is independent of the induction of the floral stimulus is indicated by the fact that even in the strain considered to be non-vernalizable so far as flowering is concerned, vernalization does have an effect in decreasing heat damage to the leaves and stipules as well as permitting the development of viable seeds.

Again, independence of heat resistance from the induction of flowering is indicated in the response of the susceptible, vernalizable variety, Unica. In this variety the first node to flower is not lowered, as would be expected from vernalized plants when these plants are subjected to a high temperature immediately following the cold treatment. Thus devernalization with respect to flowering occurs, while devernalization with respect to heat resistance is not observed under the same treatment.

Summary

The effect of a cold treatment on the response of pea plants to high temperature has been studied. It was found that there is a marked increase in heat resistance as a result of a cold treatment. The induction of heat resistance as a result of vernalization appears to be independent of the induction of the floral stimulus resulting from such a treatment. This is indicated by two observations: 1) Vernalization does have an effect in decreasing heat damage as well as effecting seed viability in a strain of peas which is non-vernalizable so far as flowering is concerned. 2) In a strain which is vernalizable, high temperature devernalizes with respect to flowering. However, vernalization markedly increases the heat resistance of this strain.

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THE POSSIBLE ROLE OF ADENOSINE TRIPHOSPHATE IN RUBIDIUM ABSORPTION AS REVEALED BY THE INFLUENCE OF EXTERNAL PHOSPHATE, DINITROPHENOL AND ARSENATE[1]

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Ion accumulation in plant cells requires aerobic respiration. According to Lundegardh's theory of anion absorption, the driving mechanism for the process is the cytochrome system which transports anions inward as electrons move outward in the steps of terminal oxidation (9). DNP (2,4-dinitrophenol)—which in animal mitochondria is known to interfere with metabolic steps involving adenosine triphosphate (ATP) (7, 8)—inhibits salt accumulation by plant tissue while maintaining or increasing the rate of respiratory oxygen consumption (11). This suggests that ATP, an energy source for many reactions, may be rather directly linked with the ion accumulation process. The present study deals with some experiments designed to obtain further evidence on the hypothesis that active salt uptake may be closely related to ATP.

If ATP is closely linked with ion accumulation, then several possible consequences may be useful in testing this hypothesis. One is that inorganic phosphate deficiency may limit the amount of ATP and thus lower ion uptake. Another possibility is that DNP depression of ion uptake is due to interference with phosphate transfers to or from ATP; the rate at which useful bond energy from ATP is made available should be related to amounts of ion accumulation. Finally the presence of ATP should be demonstrable.

Previous work has shown that Rb^+ is absorbed by excised tissues (3, 4, and 13) much as in the case of potassium and that after a more rapid initial uptake the amount of cation uptake parallels that of anion absorption (13). Also Rb^{86} appears to be a suitable tracer for K^+ in short term experiments (6). Auxin may enhance Rb^+ uptake in amounts related to the auxin induced increase in respiration (3); and, in turn, auxin activity has been reported to be related to oxidative phosphorylation (2) and, in fact, to raise the level of ATP (10).

MATERIALS AND METHODS

The tissues used for most tests were 5 mm segments of etiolated pea epicotyls, 3rd internode (Alaska var.); in addition, for some experiments, discs (approximately 1.2 mm thick × 7 mm diameter) of potato tubers (Katahdin var.), or of Jerusalem artichoke tubers were used. The pea seedlings were grown in complete darkness for 7 days. The upper 2 cm below the bud were cut into segments and used immediately. Potato or artichoke discs were aerated overnight in distilled water prior to tests.

Rubidium-86 was used as a tracer for Rb^+ and P^{32} for $H_2PO_4^-$. Specific activities were in the range of 0.01 to 0.05 μc/ml of experimental solution. Following the experimental treatment, the tissues were removed from the solution with a small sieve, rinsed briefly (about 30 sec) with distilled water, then blotted and weighed. In these experiments no attempt was made to distinguish the amount of the ion of "apparent free space" from that actively accumulated. All counting was done on dry samples which had been wet ashed with concentrated nitric acid as described previously (4). Assays of radioactive samples were made with a scaler and thin end window Geiger tube. The solutions were adjusted to pH 4.8 to 5.0 unless specified otherwise. Respiration measurements were made with a Warburg apparatus at 25° C in the salt solutions indicated, i.e., without added buffer.

The Rb H_2PO_4 was made by titrating H_3PO_4 with Rb_2CO_3 solution. In experiments in which KH_2PO_4 concentration was varied, the K^+ level was kept constant by adding the required amounts of KCl. RbCl and Rb^{86} levels were constant in all tests.

Phosphate assays were made with a colorimetric molybdate method described by Umbreit et al (14) and using a Cenco Photelometer.

RESULTS

EFFECT OF PHOSPHATE CONCENTRATION ON RUBIDIUM UPTAKE: Some experiments were conducted to see whether internal phosphate concentrations limit ion uptake and whether externally added phosphate might evoke greater Rb^+ absorption. In these experiments seedlings were grown in phosphate-deficient nutrient solutions and the 3rd internode segments excised for tests of the effect of external phosphate concentration on Rb^+ accumulation. The pea seeds used in these experiments came from plants grown in phosphate-deficient soils.

In general, the results of these experiments indicate that phosphate did not affect Rb^+ uptake ap-

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preciably. Results of one of the tests are shown in figure 1. In this test indole acetic acid (IAA) and sucrose were added to all solutions in an attempt to increase demand for phosphate. There is a suggestion in the data that at the lowest level of $H_2PO_4^-$ in the tissue (14.5 μM per g initial fresh weight) slightly less Rb^+ absorption occurs. Several unsuccessful attempts were made to obtain pea seedling tissue with less $H_2PO_4^-$ content, e.g., by excising cotyledons and by growing excised epicotyls in sucrose solution. These trials gave appreciably less stem growth and led to the surmise that the minimum amount of phosphate required for growth of tissue is also quite adequate for Rb^+ uptake.

It is significant that Rb^+ uptake was essentially the same in these experiments whether $H_2PO_4^-$ or Cl^- ion predominated. One might have expected that Rb^+ uptake would proceed at a faster rate with Cl^- than with $H_2PO_4^-$, but any difference was small or insignificant under the conditions of these experiments.

PHOSPHATE ABSORPTION AND EXCHANGE: The time courses of absorption and exchange (approximate values) of P^{32} labeled $H_2PO_4^-$ are shown in figure 2. In 2 such time course experiments a lag was noted during the first 8 hours in net accumulation of $H_2PO_4^-$, i.e., no net increase in tissue phosphate occurred; however labeled phosphate was taken up. Thus exchange of internal and external phosphate was taking place prior to increased net accumulation which followed. Approximations of this exchange in percentage were made as:

$$\frac{\text{Labeled } H_2PO_4^- \text{ uptake}}{\text{Total } H_2PO_4^- \text{ in tissue}} \times 100$$

In time course experiments with Rb^+ there was no lag in accumulation of this ion (in single salt solution); however, it is presumed to exchange with internal K^+ and thus the values reported here primarily reflect influx amounts not corrected for any efflux. It is of interest to note that, aside from exchange, $H_2P^{32}O_4^-$ uptake is essentially linear for 48 hours and internal labeling is essentially complete at the end of this period.

EFFECT OF DNP ON ABSORPTION AND RETENTION OF Rb^+ AND $H_2PO_4^-$: DNP has been shown to profoundly affect phosphate metabolism and to result in disruption of various energy consuming activities of living cells such as growth and ion accumulation. The action of DNP appears to be complex since some results suggest that it prevents ATP formation by uncoupling phosphorylation from aerobic respiration (8) possibly because it induces dephosphorylation (7). The latter data further indicates a possible degeneration of protoplasmic structure.

An interesting effect of DNP in this connection is that it may increase O_2 consumption while depressing growth and ion uptake (11). Thus it would appear that the cytochrome system proceeds at a faster pace while working energy of the cell is blocked. Under these conditions, if the Lundegardh theory of anion respiration and salt absorption holds, then salt

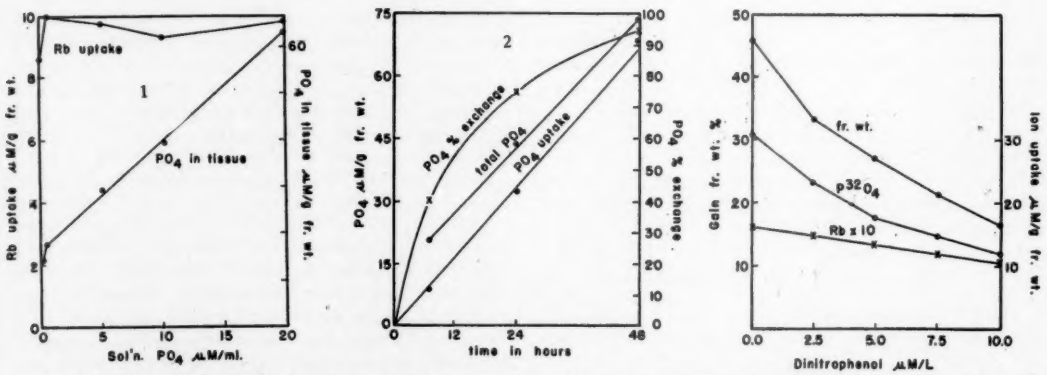


FIG. 1 (*left*). Rb^+ uptake by pea segments at various concentrations of phosphate. RbCl concentration 5 m eq/l. Duration of expt. 24 hr. All soln. contained 1 ppm indoleacetic acid and 1 % sucrose.

FIG. 2 (*center*). Time course of P^{32} labeled phosphate absorption showing total tissue phosphate content and percentage of exchange (of labeled phosphate with tissue phosphate). External solution 10 mM KH_2PO_4 per liter.

FIG. 3 (*right*). Inhibition of absorption of PO_4 and of Rb^+ by varying concentrations of DNP (2,4-dinitrophenol) and the effect of DNP on total phosphate retained by the tissue. External solution 10 μM KH_2PO_4 plus 0.5 μM RbCl per ml. The PO_4 curve represents one series, P^{32} labeled, the Rb^+ curve a parallel series, Rb^{86} labeled. Duration of experiment 24 hr.

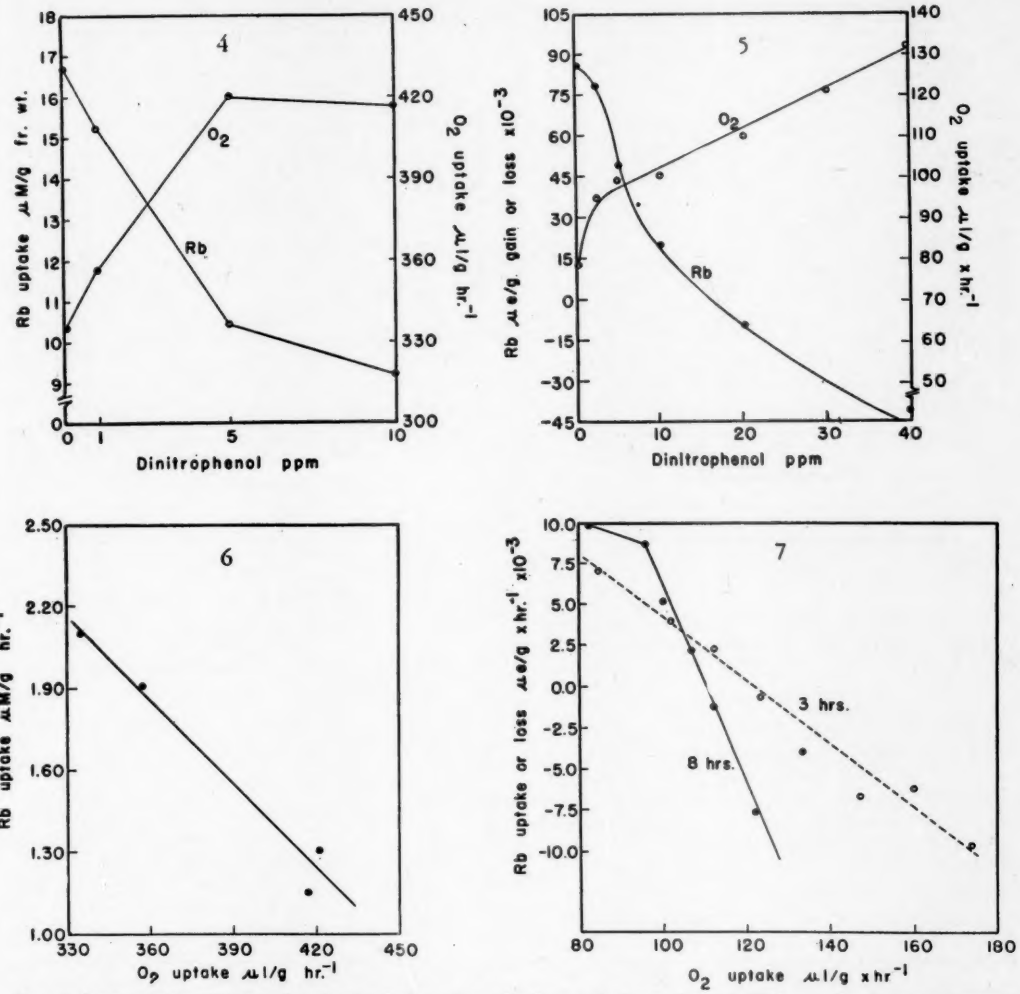
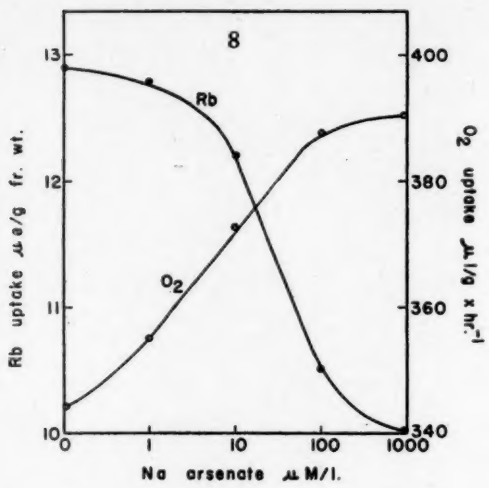


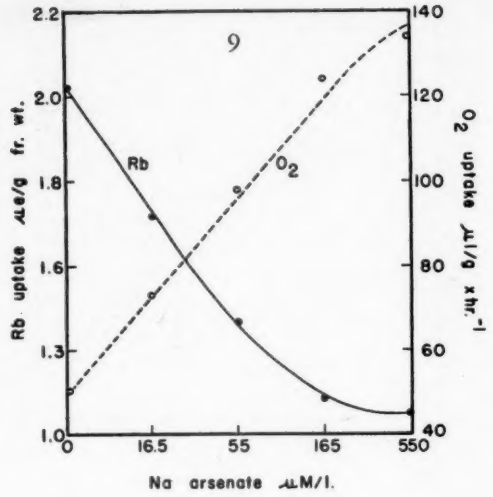
FIG. 4 (*upper left*). Dinitrophenol stimulation of respiration and depression of Rb^+ uptake in pea segments. Time of treatment 8 hrs. RbH_2PO_4 conc. 10 mM/l.

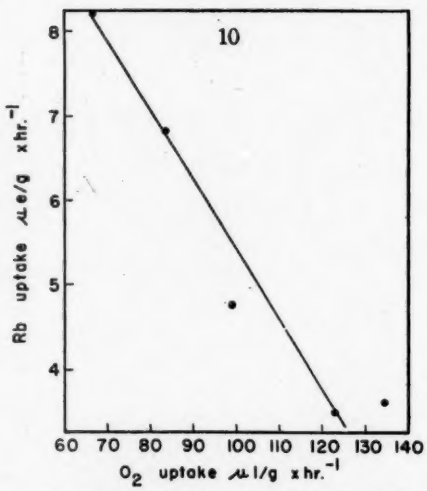
FIG. 5 (*upper right*). Dinitrophenol stimulation of respiration and inhibitions of Rb^+ uptake or induction of Rb^+ loss from tissue in potato discs. Discs were allowed to absorb Rb^+ 3 hrs prior to DNP addition. Time of DNP treatment 8 hrs. RbCl conc. 140 μM/l.

FIG. 6 (*lower left*). Data of experiment on pea stem (fig 4) showing the inverse linear relation of Rb^+ uptake to O_2 consumption when the latter is influenced by a range of DNP concentration.

FIG. 7 (*lower right*). Data on experiment with potato discs showing that Rb^+ uptake or loss is inversely related to O_2 consumption when the latter is influenced by DNP over a small concentration range.







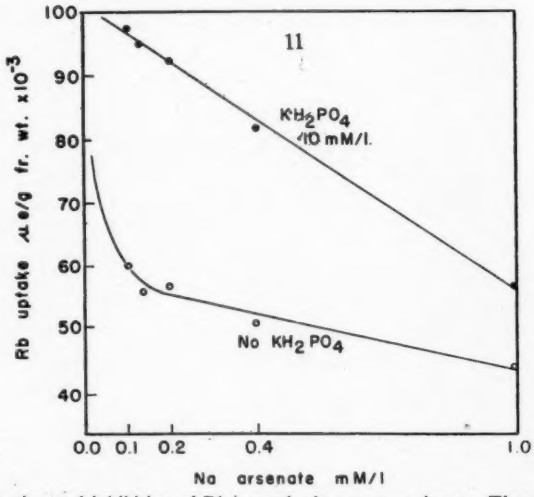


FIG. 8 (*upper left*). Arsenate stimulation of respiration and inhibition of Rb^+ uptake in pea stem tissue. Time of treatment 5 hr. RbCl conc. 10 mM/l.

FIG. 9 (*upper right*). Arsenate stimulation of respiration and inhibition of Rb^+ uptake in Jerusalem artichoke tissue discs. Time of treatment 22 hr. RbCl conc. 1 mM/l.

FIG. 10 (*lower left*). Data on experiment (fig 10) with Jerusalem artichoke tissue showing that Rb^+ uptake may be inversely related to O_2 consumption when the latter is increased by Na arsenate over a certain concentration range.

FIG. 11 (*lower right*). The effect of phosphate on arsenate inhibition of Rb^+ uptake in artichoke tissue. Time 3.5 hr. Rb^+ conc. 56.4 μ eq/l.

uptake might be expected to proceed at a faster rate, since, according to Lundegardh's theory, anions pass in via the cytochrome system as electrons move outward in the terminal oxidation system. Lundegardh's explanation of this apparent contradiction is that oxidation of cytochrome b is reversibly coupled to formation (or decomposition) of ATP; that DNP inactivates cytochrome b; that cytochrome b is involved with ATP in maintaining normal protoplasmic structure; and that high energy phosphate may be required in the formation of special carriers between b and the final site of accumulation (9). An alternative hypothesis is that ATP is required in salt absorption but that the cytochromes are not directly involved.

In a number of experiments testing the effect of DNP on Rb^+ uptake, it has been found that absorption is depressed. In general, there appears to be an inverse linear relation between the amount of Rb^+ uptake and the concentration of DNP under conditions in which the tissue is not obviously damaged by DNP. This effect is illustrated by figure 3 which shows the influence of DNP concentration on Rb^+ absorption by pea stem tissue and, in a parallel series of flasks, the influence on phosphate uptake and tissue content. Here the Rb^+ absorption was depressed in a linear fashion, whereas the response of phosphate absorption was a more complex function of DNP inhibition. However, there was a qualitative correlation of phosphate and Rb^+ uptake since absorption of each was depressed over the same concentration range of DNP.

Subsequent experiments have been made on absorption of Rb^+ and K^+ from solutions of RbCl, KCl, and mixtures of these using both Rb^{86} and K^{42} tracers. These indicate that Rb^{86}, within reasonable limits acts as a tracer for K^+ which is in accord with a previous report (6). Thus the Rb^+ absorption curve in figure 3 essentially reflects the K^+ absorption by the tissue.

The percentage gain in fresh weight in this experiment (fig 3) was closely parallel to the phosphate curves. At zero DNP the tissue gained 45 % in fresh weight and at 10 μM DNP, 17 %.

RELATION OF Rb^+ ABSORPTION TO INCREASED O_2 CONSUMPTION INDUCED BY DNP: In the effective concentration range of DNP, lower levels, about 10^{-5} to 10^{-6} molar, induce increased O_2 consumption in many plant and animal tissues; this effect reaches a maximum and at higher concentrations respiration is depressed (12).

The writer has investigated the effect of DNP on salt uptake and respiration of pea stem, potato and Jerusalem artichoke tissues. In each of these under appropriate conditions DNP induces an enhancement of respiration which is accompanied by a reduction of Rb^+ absorption. This is well illustrated by one of several tests with pea stem tissue, (fig 4), and by one of the experiments with potato discs, (fig 5); in each of these figures both oxygen consumption and Rb^+ uptake are shown and may be noted to be inverse to one another. This is in accord with the data of Robertson, et al (11) for carrot tissue.

If it is assumed that DNP interferes with the amount of high energy phosphate available for useful work, then the DNP effect at low concentration range in tissue may be proportional to the amount of increase in O_2 consumption induced by DNP. This appears to be the case for Rb^+ uptake and the DNP induced O_2 consumption by pea stem and potato tissue as shown in figures 6 and 7. Similar data were obtained with Jerusalem artichoke and carrot tissue.

EFFECT OF ARSENATE ON RESPIRATION AND UPTAKE OF Rb^+: Like DNP, arsenate interferes with phosphate metabolism and also may induce increased O_2 consumption in respiration. Arsenate is an effective inhibitor of growth (1) and ion uptake (5).

The action of arsenate on Rb^+ uptake and respiration is remarkably similar to that of DNP. The effect of a rather wide range of arsenate concentration on Rb^+ absorption and respiration by pea stem tissue and Jerusalem artichoke tissue is shown in figures 8 and 9 respectively. As with DNP treatment, terminal oxidation appears to be uncoupled from oxidative phosphate transfers. Under certain conditions, at least, Rb^+ uptake has an inverse linear relation to the increased O_2 consumption induced by arsenate as shown in an experiment with artichoke tissue (fig 10).

Addition of external phosphate greatly reduces or reverses arsenate inhibition of Rb^+ uptake (fig 11).

DISCUSSION

The data indicate that Rb^+ absorption is inversely related (probably proportionally) to the enhancement of O_2 consumption induced by DNP or arsenate. This may be construed as indicating a possible relation of ion uptake to oxidative phosphorylation or dephosphorylation. The lowest levels of DNP or arsenate which enhanced respiration also depressed Rb^+ uptake. This evidence allows the interpretation that the action of DNP may be to induce hydrolysis of ATP in a non-useful reaction rather than to block ATP formation. This is consistent with mitochondrial studies (7). This interpretation is contingent upon the ideas that the availability of phosphate acceptors, e.g., ADP, regulate aerobic respiration and that the induced increase in O_2 consumption with DNP is that amount tending to maintain the normal supply of high energy phosphates, e.g., ATP. Alternatively if ATP remained constant, and its energy expenditure were not impeded, ion uptake should not be depressed by threshold concentration levels of DNP; this was not the case. Arsenate appears to act in a manner similar to that of DNP.

These results appear not to conflict with Lundegardh's present concept of ion uptake since the cytochrome system is apparently coupled with oxidative phosphorylation in a manner which may require ATP energy in the sequence of steps leading to accumulation. It is interesting to note, however, that a theory

of ion absorption (not involving the cytochrome system) based on ATP utilization may equally well explain the data interpreted as indicating absorption via the cytochrome system.

Summary

Rubidium absorption and respiration of excised etiolated pea stem, potato and Jerusalem artichoke tuber were studied as influenced by inorganic phosphate, dinitrophenol (DNP) and arsenate. Within the limits of the present study the effect of added phosphate on Rb^+ uptake was negligible. DNP and arsenate, within the concentration range used, gave enhanced aerobic respiration, but depressed Rb^+ uptake. Under these conditions Rb^+ uptake was inversely related to O_2 consumption. The same concentration range of DNP enhancing respiration also depressed Rb^+ (and $H_2PO_4^-$) uptake. This is consistent with the hypothesis that DNP and arsenate block ion uptake by interfering with a necessary high energy phosphate hydrolysis.

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RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE ACTIVITY IN RELATION TO PHOTOSYNTHESIS BY INTACT LEAVES AND ISOLATED CHLOROPLASTS [1, 2]

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Arnon et al (4) have shown that illuminated spinach chloroplasts can carry out photosynthetic phosphorylation or can photosynthetically fix and reduce CO_2. There has been considerable interest as to the exact relationship between these processes and normal photosynthesis. The reported maximum rates of photosynthetic phosphorylation satisfy the requirements of currently accepted theories on the energy requirements of photosynthesis (2, 9), but no satisfactory agreement between photosynthetic CO_2 fixation by isolated chloroplasts and by intact leaves has yet been established. Thus Gibbs and Cynkin (8) reported that the rates of the light dependent uptake of CO_2 by spinach chloroplasts were very low when compared with rates of photosynthetic phosphorylation or with photosynthesis by intact leaves. To establish a quantitative relation between photosynthesis by isolated chloroplasts and intact leaves, both processes were measured under similar experimental conditions. The results are related to the activity and intracellular distribution of RuDP[3] carboxylase, a key enzyme in CO_2 fixation (5, 7).

MATERIALS AND METHODS

Pea leaves were harvested from 11 to 15 day old pea plants (*Pisum sativum* var. Laxton's Progress), which were grown under greenhouse conditions. In some experiments spinach leaves were used. The spinach plants (*Spinacia oleracea* var. Old Dominion) were raised in a growth chamber by hydroponic techniques.

Pea or spinach chloroplasts were prepared in 0.35 M NaCl according to Allen et al (1). Determination of the rate of CO_2 assimilation by illuminated chloroplasts was also based on their procedure. The reaction was carried out in Warburg flasks, which were placed in a water bath maintained at 13.5° C and illuminated from below by white light. The intensity at the bottoms of the flasks was approximately 1500 ft-c. The flasks were flushed with nitrogen for 5 minutes and after temperature equilibration the reaction was started by tipping 20 μC $NaHC^{14}O_3$ (0.2 μM) from a side arm. The reaction was normally allowed to proceed for 15 minutes. The $C^{14}O_2$ fixed was determined as described by Allen et al (1).

Photosynthesis by pea leaves was measured by a similar procedure. Pea leaves were floated adaxial surface down in M/100 Tris buffer, pH 7.4, contained in a Warburg flask, and $NaHC^{14}O_3$ was added from a side arm as above after flushing with nitrogen and allowing for temperature equilibration. In some experiments the buffer was omitted and the C^{14} was administered as $C^{14}O_2$ by using vessels with twin "Siamese" side arms. The $C^{14}O_2$ was liberated by the addition of 0.3 ml of 0.1 *N* HCl from one of the twin side arms into the $NaHC^{14}O_3$ contained in the other.

The method of Stocking (15) was followed for the fractionation in nonaqueous media of lyophilized pea or spinach leaves. Three fractions of different densities were separated, a fraction less dense than 1.34, a fraction with a density between 1.34 and 1.40, and a fraction more dense than 1.40. The fractions were dried in vacuo at room temperature and extracted with M/100 phosphate buffer, pH 6.5. Enzymatic assays were carried out on the centrifuged extracts.

FRACTIONATION IN AQUEOUS MEDIA: The leaves were ground in 0.35 M NaCl, 0.5 M sucrose or water, using a glass pestle and mortar. The ground material was filtered through 4 layers of muslin and the filtrate was centrifuged at 1,000×G for 6 minutes. The supernatant from the centrifugation was recentrifuged at 25,000 × G for 15 minutes. The two precipitates were resuspended in the original extraction medium (1,000 × G and 25,000 × G fractions respectively). When the leaves were extracted with 0.35 M NaCl, the 1,000 × G fraction was hence equivalent to chloroplasts prepared according to Allen et al (1). Enzymatic assays were carried out on the two particulate fractions and the final cell supernatant.

RuDP carboxylase was routinely assayed by the method of Fuller and Gibbs (7), except that the total $C^{14}O_2$ fixed into a 50 % alcohol extract of the reaction mixture was taken as an index of the activity of the enzyme. At least 90 % of this activity was recoverable in PGA by analysis on paper chromato-

[1] Manuscript received April 27, 1959.

[2] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[3] Abbreviations used in this paper: DNA for deoxyribonucleic acid; EDTA for ethylenediamine tetra-acetate; PGA for 3-phosphoglyceric acid; RuDP for ribulose 1,5-diphosphate; Tris for tris(hydroxymethyl) aminomethane; dpm for disintegrations per minute; chl. for chlorophyll.

grams. Isocitric and glucose-6-phosphate dehydrogenases were assayed spectrophotometrically by the reduction of triphosphopyridine nucleotide in the presence of their respective substrates. Malic dehydrogenase also was assayed spectrophotometrically by the oxidation of reduced diphosphopyridine nucleotide in the presence of oxalacetate.

Chlorophyll was determined according to Arnon (3). DNA was determined by measuring the deoxyribose content (6) of a 5 % perchloric acid extract (70° C for 20 min) of the material to be assayed, after prior extraction of acid soluble and lipid phosphates.

Results

$C^{14}O_2$ Assimilation by Illuminated Chloroplasts and Intact Leaves: With 10 different pea chloroplast preparations the rate of photosynthetic fixation of $C^{14}O_2$ averaged 1.2×10^7 dpm/mg chl./hr. This rate was higher than rates obtained with spinach chloroplasts prepared in an identical manner. Under the same conditions of light, temperature and CO_2 concentration, whole pea leaves photosynthetically assimilated $C^{14}O_2$ (administered as $NaCH^{14}O_3$) at the rate of 1.4×10^8 dpm/mg chl./hr. Slightly less than double this rate was obtained if the C^{14} was fed as $C^{14}O_2$ in the gas phase. Thus under similar assay conditions, the rate of photosynthesis by isolated chloroplasts was approximately either 10 % or 5 % of the rate of photosynthesis by intact leaves. The photosynthetic rates of leaves were below the maximum rate of photosynthesis because of the low temperature and low CO_2 concentration (<0.01 %). That the $C^{14}O_2$ fixation was a true measure of the rate of photosynthesis under the conditions employed was indicated by comparative experiments between $C^{14}O_2$ fixation and oxygen production. If the $C^{14}O_2$ was supplemented with $C^{12}O_2$ to give a final concentration of 1 % CO_2 by volume, then the rate of CO_2 uptake agreed with rates based on the photosynthetic production of oxygen using a "CO_2 buffer" system (10), in which the CO_2 in the gas phase was maintained at 1 %. Increasing the bicarbonate concentration increased the CO_2 uptake by isolated chloroplasts.

Distribution of RuDP Carboxylase: Since it was not found possible to demonstrate photosynthesis by isolated chloroplasts at the same level as that carried out by the intact leaf, an explanation for this discrepancy was sought. Relevant information was elicited from a study of the activity and distribution of RuDP carboxylase. This enzyme is considered to be the primary site of CO_2 fixation in photosynthesis (5). Its widespread occurrence in photosynthetic organisms and its disappearance during etiolation (7) strongly indicates an intimate association with chloroplasts and photosynthesis.

Further evidence for the chloroplastic origin of RuDP carboxylase was obtained by fractionation of pea leaves in nonaqueous media (15). Since the entire fractionation was carried out in the absence of water after lyophilization of the leaves, the possible leaching of enzymes from their original structural units in the cell was avoided. The results of these studies are shown in table I. The RuDP carboxylase distribution paralleled the distribution of chlorophyll. Stocking (15) has shown that the least dense fraction contained the highest proportion of chloroplasts. This was confirmed by microscopic examination. Nearly 60 % of the RuDP carboxylase activity was found in the lightest fraction. On the other hand enzymes such as isocitric dehydrogenase and malic dehydrogenase, which are associated mainly with the mitochondrial fraction in pea leaves (14), occurred principally in the 1.34 to 1.40 fraction. This fraction was also high in glucose-6-phosphate dehydrogenase. In rabbit liver and kidney this enzyme is associated with very small particles which may be sedimented between 105,000 and $144,000 \times G$ (12), although it has also been reported present in mitochondria from etiolated pea plants (13). The DNA estimation showed that the chloroplast fraction was also relatively low in nuclear material. Similarly, in spinach leaves, 70 % of the RuDP carboxylase was located in the fraction having a density less than 1.34. These distribution studies then provide additional evidence for the importance of RuDP carboxylase in the metabolism of the chloroplast.

The distribution of RuDP carboxylase in cell fractions which were isolated in various aqueous media was quite different. As described in the meth-

Table I.

Distribution of Some Enzymatic Activities in Pea Leaf Fractions Isolated in Nonaqueous Media

Substances assayed for	% Distribution in Fractions		
	Density of fractions isolated		
	<1.34	1.34-1.40	>1.40
Isocitric dehydrogenase	21	44	35
Malic dehydrogenase	27	45	28
Glucose-6-phosphate dehydrogenase	28	40	32
RuDP carboxylase	56	40	4
Chlorophyll	56	37	7
DNA	11	78	11

The reaction mixture for the dehydrogenase assays contained in addition to the enzyme Tris buffer, pH 7.4 (0.05 M), $MgCl_2$ (0.004 M), pyridine nucleotide (100 μg/ml), and substrate (0.007 M). The change in optical density at 340 mμ was recorded. The reaction mixture for RuDP carboxylase contained in addition to the enzyme potassium phosphate buffer, pH 6.5 (0.05 M), RuDP (0.1 μM/ml) and $NaHC^{14}O_3$ (0.1 μM/ml containing 10 μC). The reaction was carried out in a stoppered 12 ml centrifuge tube. After 1.5 minutes the reaction was stopped by adding boiling 80 % alcohol.

ods section, three fractions were separated after extraction of the leaves with 0.35 M NaCl. The 1,000 × G (chloroplast) fraction when examined microscopically consisted mainly of whole chloroplasts. The 25,000 × G fraction contained some whole chloroplasts, but consisted mainly of chloroplast fragments and small particles such as mitochondria. Both fractions contained starch grains. The final cell supernatant was virtually free of chlorophyll containing material. An assay of the 3 fractions for their RuDP carboxylase activity showed that the 2 particulate fractions each contained only about 1 % of the total activity (table II). If, however, the cofactors $MgCl_2$, glutathione and EDTA were added to the reaction mixture in the concentrations used by Weissbach et al (16) to give optimal activity, 9 % to 13 % of the RuDP carboxylase activity was found in the 1,000 × G fraction. Presumably the supernatant already contained cofactors. The figures in the third column of table II show the amount of leaf chlorophyll released during the extraction process and are thus indicative of the severity of grinding. Gentle or thorough grinding with a glass pestle and mortar did not appear to affect appreciably the RuDP carboxylase activity of the chloroplast fractions. On the other hand, thorough grinding with a Ten Broeck hand model glass homogenizer, which produced greater breakage of the chloroplasts as evidenced by the chlorophyll distribution (table II), resulted in decreased carboxylase activity in the chloroplast fraction. If NaCl was replaced by 0.5 M sucrose, greater breakage of the plastids resulted and less carboxylase was retained in the chloroplast fraction. If the leaves were extracted with water, over 99 % of the carboxylase activity was released into solution.

Spinach leaves were fractionated and assayed for RuDP carboxylase in the same way. The particulate fractions appeared to contain a greater percentage of the carboxylase activity than was found for pea leaves. It was thought that these results were misleading because of the presence in spinach extracts of a water soluble inhibitor for RuDP carboxylase reported by Weissbach et al (16). The activity of the supernatant fraction would then be too low. When expressed on a chlorophyll basis, the carboxylase activity of spinach homogenates was only about one-third that found for pea homogenates. If the carboxylase activity of the spinach chloroplast fraction was assayed indirectly by determining the activity of a homogenate before and after the removal of the chloroplasts, then less than 10 % of the activity was found in the chloroplast fraction.

RuDP Carboxylase Activity and Rate of Photosynthesis: As shown in table II the retention of RuDP carboxylase in chloroplasts prepared in NaCl

Table II.

Distribution of RuDP Carboxylase in Fractions from Pea Leaves

Expt.	Medium	% Leaf chlorophyll released during grinding*	Fraction	% Distribution of carboxylation enzyme**	% Distribution of chlorophyll
1	NaCl	47	1000 × G	0.9	41
			25,000 × G	1.3	56
			Supernatant	97.8	3
2	NaCl	43	1000 × G	9.0	38
			25,000 × G	1.8	59
			Supernatant	89.2	3
3	NaCl	49	1000 × G	11.2	44
			25,000 × G	1.1	52
			Supernatant	87.7	4
4	NaCl	86	1000 × G	12.8	37
			25,000 × G	2.5	61
			Supernatant	84.7	2
5	NaCl	96	1000 × G	3.1	20
			25,000 × G	2.1	73
			Supernatant	94.8	7
6	Sucrose	38	1000 × G	7.2	29
			25,000 × G	2.1	66
			Supernatant	90.7	5
7	Water	...	1000 × G + 25,000 × G	0.7	
			Supernatant	99.3	

* The pea leaves were ground with a glass pestle and mortar (except in experiment 5 in which a glass hand homogenizer was used).

** $MgCl_2$ (10 μM/ml), glutathione (6 μM/ml), and EDTA (0.06 μM/ml) were present in the assay reaction mixtures (except in experiment 1). For the other components of the reaction mixtures see table I. The total activity of the carboxylation enzyme in leaf extracts is given in the text and table III.

is about 10 %. The rate of photosynthesis by isolated chloroplasts compared with photosynthesis by intact leaves is also about 10 %. It is possible that the rate of CO_2 assimilation by isolated chloroplasts (or intact leaves under the conditions employed here) is limited by their RuDP carboxylase activity, i.e., that the RuDP carboxylase activity is a direct measure of photosynthetic activity. This hypothesis is supported by the fact that during the photosynthetic assimilation of $C^{14}O_2$ by photosynthetic cells, the amount of PGA (the known immediate product of the carboxylation reaction) is extremely low in all but very short term feeding experiments. In the present experiments it was demonstrated that isolated pea chloroplasts fix $C^{14}O_2$ into a similar spectrum of compounds that have been found in numerous studies of $C^{14}O_2$ fixation by photosynthetic cells. Thus the reductive and energy requiring reactions following the formation of PGA do not appear to be limiting if the reactions are carried out in saturating light. This correlates well with the observed high rates of formation of photosynthetic reductive power and energy by partially purified chloroplast preparations (2, 9).

In order to demonstrate a further quantitative relation between photosynthetic rate and the RuDP carboxylase, pea leaves were extracted by grinding with dilute M/100 Tris buffer, pH 7.8; the extract was centrifuged at 25,000 × G for 15 minutes, and the supernatant was tested for its ability to fix $C^{14}O_2$ in the dark and in the presence of RuDP. The optimum pH and cofactor concentrations as found by Weissbach et al (16) were used; otherwise conditions were kept as close as possible to the conditions used for measuring the uptake of $C^{14}O_2$ either by leaves or by chloroplast preparations. Values up to 2.1×10^5 dpm/mg chl./hr have been obtained; i.e., under optimum conditions the ability of the extracts to fix CO_2 in the dark was approximately the same as the ability of the leaves to fix CO_2 photosynthetically. If the cofactors $MgCl_2$ and glutathione were replaced by $MnCl_2$ and ascorbate at the concentrations employed by Allen et al (1), then the carboxylase activity of the extracts fell to approximately 60 %. If all cofactors were omitted the activity fell to 10 % or less. Hence the observed low photosynthetic activity of isolated chloroplasts can be accounted for by the amount and activity of their RuDP carboxylase. For convenience, the more important rates discussed above are listed in table III.

The correspondence between the photosynthetic ability of isolated chloroplasts and their RuDP carboxylase activity has also been shown by direct experimentation. Pea chloroplasts were prepared as usual and were resuspended in a small volume of 0.35 M NaCl. The suspension was divided into two equal portions. One part was diluted with NaCl and the other was diluted with an equal volume of water to produce broken chloroplasts. The photosynthetic activity of the whole chloroplasts was compared with the RuDP carboxylase activity of the chloroplasts (i.e., the dark fixation of CO_2 by the broken chloroplasts in the presence of RuDP). The same amount of chlorophyll was present in each reaction mixture. The results are shown in table IV. The $C^{14}O_2$ fixed by the whole chloroplasts in the light was the same as the ability of the broken chloroplasts to fix $C^{14}O_2$ in the presence of RuDP, in either the light or the dark. That the water treated pea chloroplasts were broken was shown by their inability to fix $C^{14}O_2$ in light. The small increase in the dark fixation of $C^{14}O_2$ by whole chloroplasts in the presence of added RuDP was probably due to residual carboxylase from the cell supernatant. The RuDP apparently could not penetrate the membrane of the intact chloroplast.

TABLE III.

PHOTOSYNTHETIC ACTIVITY OF CHLOROPLASTS AND LEAVES

	$C^{14}O_2$* FIXED IN DPM/MG CHL./HR
Photosynthesis Whole Leaves	1.4×10^8 2.5×10^{8}**
RuDP Carboxylase Activity of Leaves	2.1×10^8
Photosynthesis Chloroplasts	1.2×10^7

* Specific Activity of $NaHC^{14}O_3$ fed equals 10 μC/0.1 μM.

** C^{14} fed as $C^{14}O_2$ instead of $NaHC^{14}O_3$.

TABLE IV.

PHOTOSYNTHETIC ACTIVITY OF ISOLATED PEA CHLOROPLASTS COMPARED WITH THEIR RuDP CARBOXYLASE ACTIVITY

CONDITION OF CHLOROPLASTS	LIGHT OR DARK	RuDP (0.2 μM)	TOTAL COUNTS FIXED × 10^4 DPM
Whole	D	−	14
"	L	−	177
"	D	+	21
Broken	L	−	8
"	D	+	191*
"	L	+	184*

Reaction mixture contained whole or broken chloroplasts, NaCl (1.05 μM, whole chloroplasts only), Tris buffer, pH 7.4 (40 μM), $MnCl_2$ (2 μM), potassium ascorbate (10 μM) and $NaHC^{14}O_3$ (0.2 μM containing 20 μC). Final volume 3.0 ml. Temperature 13.5° C.

* C^{14} fixed into PGA only.

DISCUSSION

The experiments presented above emphasize the importance of RuDP carboxylase in photosynthesis. Not only is it a primary site of CO_2 fixation, but also under certain conditions such as low CO_2 concentration, the carboxylase reaction appears to limit the

photosynthetic rate. In such cases it is an assay for photosynthesis. As shown by the fractionations using nonaqueous extraction solvents, the RuDP carboxylase is associated with the chloroplast in higher plants. This has already been strongly implicated by the work of Fuller and Gibbs (7). The techniques that are currently being used for the isolation of chloroplasts, such as extraction in 0.35 M NaCl or 0.5 M sucrose, are inadequate for the preservation of chloroplast structure and result in the loss of 90 % or more of the RuDP carboxylase from the plastids. These losses of RuDP carboxylase can satisfactorily account for the observed low rates of photosynthesis by isolated chloroplasts. Cofactors and other enzymes are also probably lost. Hence it must be pointed out that the study of the mechanisms of CO_2 fixation by chloroplasts isolated in salt or sucrose solutions, should take into account that only a small fraction of the chloroplasts is functionally intact.

The inability of RuDP to penetrate intact chloroplasts (table IV) indicates that major damage must be occurring to the chloroplast membrane during isolation of the plastids in aqueous media, since if RuDP is unable to penetrate the intact plastid, it is unlikely that RuDP carboxylase is easily lost unless some breakage of the membranes occurs. The presence of starch grains may be a major cause of membrane breakage as the grains appear to break free easily from the chloroplasts during grinding of the leaves and centrifugation of the homogenate. The fact that 90 % of the RuDP carboxylase is so easily lost from the chloroplasts suggests that the RuDP carboxylase originates in the proteins of the chloroplasm (11) rather than in the grana. Enzymes other than the carboxylase have not been tested for their ease of extraction from pea chloroplasts prepared in aqueous media. Stocking (15) demonstrated by his nonaqueous fractionation method that phosphorylase is associated with the chloroplast fraction in tobacco. He found that this enzyme was lost from chloroplasts when they were isolated in aqueous media. An alternative explanation for the loss of RuDP carboxylase from the chloroplast is that it is normally absorbed on the surface of the chloroplast. There is no evidence for this and it is very unlikely if, as stated by Weissbach et al (16), the enzyme constitutes 5 % to 10 % of the total soluble protein of spinach leaves.

In contrast to the above there is an apparent ability of the remaining few "intact" chloroplasts to make maximum use of their RuDP carboxylase to fix CO_2 and to convert it photosynthetically via PGA to a series of compounds which are intermediates in normal photosynthesis. These chloroplasts appear to be exhibiting true photosynthesis.

Summary

Rates of photosynthesis by chloroplasts isolated from pea leaves and by intact pea leaves have been measured under the same conditions of light, temperature and CO_2 concentration. The conditions were chosen to approximate those previously employed for measuring photosynthesis by whole chloroplasts. When expressed on a unit chlorophyll basis, the isolated chloroplasts had 5 % to 10 % the photosynthetic activity of the leaves.

About 10 % of the total RuDP carboxylase of leaves was retained in chloroplasts isolated in 0.35 M NaCl. Fractionation in organic media indicated that this enzyme is normally associated with the chloroplasts in intact leaves. The capacity of the total leaf RuDP carboxylase to fix CO_2 in the presence of RuDP was of the same order as the capacity of the leaves to fix CO_2 photosynthetically. Similarly, the CO_2 fixing capacity of RuDP carboxylase from isolated chloroplasts was approximately the same as their photosynthetic activity.

Under the assay conditions used RuDP carboxylase appeared to limit the photosynthesis of both isolated chloroplasts and intact leaves. The loss of RuDP carboxylase from the chloroplasts during their isolation in 0.35 M NaCl can account for their observed low rates of photosynthesis.

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ESTIMATED CONTRIBUTIONS OF ROOT AND SHOOT TO THE NICOTINE CONTENT OF THE TOBACCO PLANT[1]

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In earlier papers (1, 3, 4), we have described patterns of alkaloid accumulation in reciprocal grafts of tobacco with tomato. The results have been interpreted to mean that the tobacco shoot possesses a low capacity for alkaloid production and that the root system is the origin of most of the alkaloid found in the intact plant. However, existing data do not permit assignment of specific values to these capacities. For this purpose, there is need for data obtained from grafted as well as intact (nongrafted) plants which have been grown under similar conditions and have exhibited similar rates of growth and development. It may then be assumed that the alkaloid content of the grafted shoot affords a reasonable estimate of the contribution of the intact shoot to its own alkaloid content. The present paper utilizes this assumption to provide first approximations of the relative alkaloid yielding capacities of root and shoot of 3 varieties, representing 2 species, of Nicotiana.

This approach has been extended to include the case in which the apical bud of the tobacco shoot is removed (topping) while the plant is still in the vegetative stage of development. Tso and Jeffrey (6) and Solt and Dawson (4) have shown that the alkaloid content of tobacco scions grown on tomato rootstocks may be greatly increased by topping. Such an effect is also obtained as a result of the topping of tobacco in commercial plantings.

MATERIALS AND METHODS

PLANT MATERIALS: Two species of Nicotiana were employed in these experiments, *N. tabacum* L. vars. Turkish and Connecticut Shade No. 49, and *N. rustica* L. var. gigantea. All were grown in the greenhouse during the winter and spring months of 1957 to 1958. The methods of culture, of cleft-grafting, of harvest and of preparation for assay have been described in our earlier papers (3, 4).

Tomato (*Lycopersicum esculentum* Mill. var. Marglobe) was used as rootstock. Tobacco scions were shoots cut above the 5th leaf from young plants which had grown out of the rosette stage. Nongrafted plants were stripped of the lowermost 5 leaves to compensate for similar losses by the scions. Since about 3 weeks were required to permit resumption of normal growth rates by grafted scions, the latter were harvested 3 weeks later than the non-grafted plants. The time schedule for grafting and topping is given in table I.

Plants were sampled at 2 stages of development; one just before flower bud emergence (crop 1) and the other when green seed pods were present (crop 2).

In general, 5 (sometimes 4) plants representing each type and treatment were harvested and divided separately into leaf, stem, inflorescence and, in some cases, root fractions for assay.

BASIS FOR COMPARISON: In the absence of appreciable rates of destruction the alkaloid content of an intact tobacco plant is obviously the sum of the contributions of root, stem, leaves and inflorescence, if any. The total alkaloid content of an individual plant, however, increases rapidly during growth and development, and the production of alkaloid relative to dry weight is sensitive to certain elements of the environment. For these reasons, there is some advantage to basing estimates of alkaloid yielding capacities of the different plant parts upon the total

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TABLE I

TIME SCHEDULE IN DAYS FOR GRAFTING, TOPPING AND HARVEST *

VARIETY	CROP	NONGRAFTED		GRAFTED		
		PLANTING TO TOPPING	TOPPING TO HARVEST	PLANTING TO GRAFTING	GRAFTING TO TOPPING	TOPPING TO HARVEST
Connecticut shade No. 49	1	110	22	**	**	22
	2	110	59	**	**	63
Turkish	1	115	23	87	51	27
	2	115	58	138	51	57
Rustica	1	119	31	84	58	20
	2	119	58	142	58	42

* In all cases nontopped plants were the same age at harvest as the corresponding topped plants.

** Due to premature flowering the plants were cut back near graft union and allowed to produce new shoots.

amount of alkaloid present in the individual organ, shoot or whole plant rather than upon unit dry weight.

For the purposes of the present work, therefore, the following procedure was followed: First, the total amounts of nicotine in root, stem, leaf and inflorescence of intact or topped but nongrafted plants were determined. Second, the contributions of leaves, stem and whole shoot to their own nicotine contents were estimated by assaying the corresponding parts of the grafted plants. Third, the contributions of the root system were obtained by difference between the nicotine contents of the whole plant and of the grafted shoot. Fourth, the nicotine content of leaves, stem or whole shoot of the grafted plant was divided by the nicotine content of the leaf, stem, shoot or whole plant of the non-grafted plant, respectively, in order to obtain the relative contributions of each of these parts to its own nicotine content.

It may be noted that these estimates of relative alkaloid producing capacity are not complicated by the occurrence of appreciable downward transport of alkaloid in the plant. There are, however, some relatively small errors which have been identified in the case of varieties of *N. tabacum*. The graft union is a source of quantities of alkaloid which can be only partially eliminated from the estimates by excision of the union at time of harvest. Quantities which have been transported from the union upwards to stem and leaves (4) cannot be approximated with any degree of confidence and, when included in the total alkaloid figures for these organs or for the shoot as a whole, will lead to overestimates of the alkaloid yielding capacities of these structures. In the 2nd crop of *N. tabacum* var. Turkish, a partial correction can be achieved by subtracting from the contribution of the grafted stem, the abnormally high alkaloid content (loc. cit.) of the lowermost 150 mm and substituting for this figure a quantity equal to the nicotine content of the next 150 mm section above it. Since the alkaloid contents of the 1957 (4) and 1958 (present) crops were not identical, it was assumed for purposes of making this correction that the same fraction of total alkaloid in the stem occurred in the first 150 mm above the graft union in both crops.

The data for *N. rustica* do not involve the graft union effect. It is very likely, therefore, that the estimates for *N. rustica* are more accurate than for those of the 2 varieties of *N. tabacum*. In these latter cases, however, the relative contributions of leaves, stems and shoots will be overestimated somewhat while those of the roots will be underestimated.

To ensure validity of comparison between grafted and nongrafted plants, careful attention was given to the timing of all operations (see above), to cultural treatment of the plants and to the equalizing of morphological characteristics such as number of leaves, stalk height, etc. Some of these items are detailed in tables I and II (column 5).

TABLE II

DRY WEIGHT IN G AND NUMBER OF LEAVES PER PLANT

TREATMENT	MEAN DRY WEIGHT				MEAN NUMBER OF LEAVES
	LEAVES	STEMS	INFLORESCENCE	SHOOT	
N. tabacum var Turkish (vegetative stage)					
Intact plant	29.3	22.0		51.3	30
Topped	21.4	9.0		30.4	14
Grafted	28.7	14.9		43.6	33
Grafted and topped	23.9	4.9		28.8	14
var Turkish (fruiting stage)					
Intact plant	41.4	46.7	25.4	113.0	31
Topped	43.2	20.0		63.2	13
Grafted	40.9	32.0	37.2	110.3	31
Grafted and topped	51.4	8.8		60.2	13
N tabacum var Conn No. 49 (vegetative stage)					
Intact plant	23.1	23.3		46.4	22
Topped	18.6	12.5		31.1	11
Grafted	27.4	17.3		44.7	24
Grafted and topped	22.3	12.5		34.8	10
var Conn No. 49 (fruiting stage)					
Intact plant	38.5	51.1	22.2	111.8	20
Topped	36.6	24.5		61.0	11
Grafted	48.5	56.2	43.2	147.9	21
Grafted and topped	58.2	28.9		87.0	10
N rustica var gigantea (vegetative stage)					
Intact plant	49.5	34.8		84.3	23
Topped	53.3	25.5		78.8	12
Grafted	38.3	21.7		60.0	21
Grafted and topped	43.1	21.6		64.7	12
var gigantea (fruiting stage)					
Intact plant	64.0	59.0	47.7	170.7	25
Topped	80.7	44.9		125.6	12
Grafted	38.8	34.4	41.3	114.5	22
Grafted and topped	55.1	30.0		85.1	11

STATISTICAL TREATMENT: Each figure in tables II and III represents a mean of 5 (or sometimes 4) measurements. The statistics given in table III are standard deviations. Standard errors of estimate based upon means of 5 (or 4) individual measurements were calculated and used in the following equation[2]:

$$\left[\frac{\sigma\bar{x}_1}{\bar{x}_2}\right]^2 = \left[\frac{\sigma x_1}{X_1}\right]^2 + \left[\frac{\sigma\bar{x}_2}{X_2}\right]^2,$$

to calculate standard errors of estimate of the ratios described above.

[2] $\sigma\bar{x}$ = standard deviation

$\bar{X}$ = mean

CHEMICAL METHODS: Total steam-volatile pyridine alkaloid content was estimated by methods described in earlier papers (cf. 2). The minor alkaloid are incompletely distillable from magnesium oxide with steam under our conditions. Paper chromatographic examination of the distillates indicated that our measurements refer almost entirely to nicotine. The assay figures are hence calculated as nicotine. It was thought necessary, however, to examine also the alkaloid complex of the plant materials prior to distillation so that any substantial shifts in alkaloid composition associated with a given experimental treatment would be detected.

In preparation for chromatography, 10 gm of dry leaf powder was mixed thoroughly with 10 ml of *N* HCl and extracted continuously with petroleum ether for 24 hours. The extracted residue was then removed from the Soxhlet thimble and air-dried. One ml of concentrated ammonium hydroxide was then added together with enough ethyl ether to yield a slurry. This was shaken mechanically for 3 hours in a closed flask. After filtration through glass wool, the ethereal solution of alkaloids was concentrated to low volume and applied to sheets of Whatman No. 1 filter paper previously impregnated with an acetate

TABLE III

MEAN NICOTINE CONTENTS AND THEIR STANDARD DEVIATIONS IN MG PER PLANT

TREATMENT	LEAVES	STEMS	INFLORESCENCE	SHOOT	ROOTS
		N tabacum var Turkish (vegetative stage)			
Intact plant	289 ± 69	106 ± 11		395 ± 76	
Topped	675 ± 157	128 ± 19		803 ± 170	
Grafted	1.5 ± 0.1	4.7 ± 0.7		6.2 ± 0.7	
Grafted and topped	15 ± 1.2	32 ± 9.4		47 ± 9.3	
		var Turkish (fruiting stage)			
Intact plant	452 ± 62	86 ± 32	40 ± 8.6	577 ± 86	144 ± 23
Topped	2486 ± 378	241 ± 38		2727 ± 429	315 ± 63
Grafted	2.3 ± 0.9	7.7 ± 1.8	0.2 ± 0.3	11 ± 1.7	
Grafted and topped	83 ± 12	65 ± 5.5		149 ± 16	
		N tabacum var Connecticut No. 49 (vegetative stage)			
Intact plant	148 ± 17	57 ± 6		205 ± 21	
Topped	380 ± 67	97 ± 21		477 ± 88	
Grafted	0.8 ± 0.2	4.6 ± 1.2		5.3 ± 1.2	
Grafted and topped	2.5 ± 1.0	18 ± 2.9		21 ± 3.6	
		var Connecticut No. 49 (fruiting stage)			
Intact plant	327 ± 17	72 ± 14	35 ± 12	433 ± 22	123 ± 29
Topped	1617 ± 308	223 ± 29		1840 ± 335	264 ± 25
Grafted	1.3 ± 0.5	8.5 ± 4.6	0.7 ± 0.4	10 ± 5.1	
Grafted and topped	39 ± 10	96 ± 27		135 ± 33	
		N rustica var gigantea (vegetative stage)			
Intact plant	841 ± 214	183 ± 32		1024 ± 240	
Topped	2139 ± 484	301 ± 7.0		2440 ± 485	
Grafted	1.2 ± 0.5	0		1.2 ± 0.5	
Grafted and topped	0.9 ± 0.7	0.8 ± 0.5		1.7 ± 0.8	
		var gigantea (fruiting stage)			
Intact plant	1084 ± 136	194 ± 40	257 ± 26	1534 ± 145	121 ± 20
Topped	3803 ± 392	558 ± 46		4361 ± 404	304 ± 50
Grafted	1.3 ± 0.4	trace	trace	1.7 ± 0.5	
Grafted and topped	2.3 ± 0.5	3.0 ± 0.5		5.3 ± 0.9	

buffer (pH 5.6). The alkaloids were separated on this sheet by the solvent mixture of Tso and Jeffrey (5), *tert*-amyl alcohol and acetate buffer (pH 5.6). Cyanogen bromide and *p*-aminobenzoic acid were used as spot reagents.

Results

Basis for Comparison: It may be seen in table II that the requirement for equality of growth and development of grafted and nongrafted shoots is fairly well realized in both crops of the Turkish variety.

In the fruiting stage the grafted shoots of the Connecticut 49 variety were substantially heavier than nongrafted shoots. In the comparison of nontopped plants, however, the dry weight discrepancies are largely accounted for by the inflorescence which in this variety does not contain alkaloids (4). The comparison between topped plants of this variety is complicated by the higher dry weight of the latter and hence the ratio is somewhat high.

The grafted plants of *Nicotiana rustica* weighed less than the nongrafted. When pooled data relating nicotine content to plant dry weight for this species are plotted on arithmetic coordinates, however, the curve, which is quite smooth, has already become asymptotic at the vegetative stage represented in the present work. Consequently, correct yield ratios are obtained. Such is not the case in the single comparison within the Connecticut 49 variety mentioned above, because different crops of grafted plants of this variety have failed to give the same relationship between the 2 variables.

Alkaloid Content of Plants: The quantities of nicotine contained in leaves, stems, inflorescence and roots, as well as in the shoot, are given in table III.

In agreement with the earlier work, grafting Turkish tobacco scions to tomato rootstocks reduced the nicotine content of the shoots in the fruiting stage by somewhat more than 1 order of magnitude. On the other hand, topping rooted Turkish plants increased the amount of nicotine in the shoots by 1 order of magnitude. The combined effect of grafting and topping was to increase nicotine content of the shoot by 1 order of magnitude over the effect of grafting alone, but the quantities present were still only about one-third to one-fourth as great as those found in the shoots of intact plants. These observations are noteworthy in connection with the findings reported in an earlier paper (4) where a close comparison of grafted and topped plants with intact plants was not possible. A similar pattern was obtained in the case of Connecticut Shade No. 49 variety. However, the data for *Nicotiana rustica* (table III) are somewhat different. Here the nicotine content of all grafted shoots was extremely low in comparison with that of intact shoots. The increase in nicotine due to topping was no more than 2- to 3-fold.

Relative Nicotine Producing Capacities of Shoot and Leaf: The 2 varieties of *N. tabacum* do not vary significantly with respect to relative nicotine contributions of shoot and leaf, whether topped or nontopped (table IV). It is of considerable interest to note that the shoot of the intact plant very likely produces no less than 1 % and no more than 3 % of the nicotine which it normally contains. The effect of topping is to double these figures. Somewhat lower figures are obtained when the ratio employed is the proportion of nicotine in the whole plant that is produced in the shoot.

Table IV

Relative Contributions (in %) of Leaves to Nicotine Content of Leaves and of Shoot to Nicotine Content of Shoot and of Whole Plant

Proportion of nicotine	Nontopped		Topped	
	Vegetative	Fruiting	Vegetative	Fruiting
		N tabacum var Turkish		
In shoot produced in shoot	1.6 ± 0.2*	1.9 ± 0.2	5.9 ± 0.8	5.5 ± 0.5
In plant produced in shoot		1.5 ± 0.2		4.9 ± 0.4
In leaf produced in leaf	0.5 ± 0.1	0.5 ± 0.1	2.2 ± 0.2	3.3 ± 0.3
		N tabacum var Connecticut No. 49		
In shoot produced in shoot	2.6 ± 0.3	2.3 ± 0.5	4.4 ± 0.5	7.3 ± 1.0
In plant produced in shoot		1.8 ± 0.4		6.4 ± 0.9
In leaf produced in leaf	0.5 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	2.4 ± 0.4
		N rustica var gigantea		
In shoot produced in shoot	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
In plant produced in shoot		0.1 ± 0.0		0.1 ± 0.0
In leaf produced in leaf	0.1 ± 0.0	0.1 ± 0.0	0.04 ± 0.02	0.06 ± 0.01

* Standard errors of estimate.

The situation in *N. tabacum* varieties is complicated (see above) by the production of alkaloid in the graft union and in the stem regions immediately above the graft union and by transport of this alkaloid to leaves higher on the same stem. For this reason ratios contained in table IV are too high by an unknown amount.

Such overestimation, however, is avoided in the case of *N. rustica*. In grafts between this species and tomato, the graft union and adjacent stem tissues do not produce and accumulate appreciable amounts of alkaloid. Hence, it may be assumed that translocation of nicotine from such sites to the leaves would be negligible or nonexistent. Therefore, the figures for relative nicotine production in the shoot are much smaller than is the case in varieties of *N. tabacum*. In all crops, whether topped or nontopped, the fraction of nicotine in the shoot, or in the entire plant which is produced in the shoot, remains rather closely around 0.1 %.

A 2nd approximation may be made of the relative nicotine producing capacity of the Turkish tobacco shoot by applying the correction for the unusually high alkaloid content of the stem immediately above the graft union. When this correction is applied to the figures for nontopped Turkish plants in the fruiting stage, the ratios and their standard errors of estimate have the following values. The fraction of nicotine in the whole plant, which was produced in the shoot, becomes 0.8 ± 0.1 %. This means that about 99.2 % of the nicotine content of the entire plant was produced in the root system. The corresponding figures for topped plants of the 2nd crop are 4.9 ± 0.4 % produced in the shoot and about 95.1 % produced in the roots. For reasons already given, both sets of figures must be regarded as maximal for the fraction produced in the shoot and minimal for the fraction produced in the root. This effect would be far more serious in the topped plants than the nontopped plants.

Estimates of relative amounts of nicotine in the leaf, which were produced in the leaf are also given in table IV. These figures represent upper limits for the reasons already given. The degree of overestimation would again be more serious in the case of the topped plants. In the nontopped plants it is quite clear, however, that the leaves produce considerably less than 1 % of their nicotine content at any given time. The figure may approach 0.1 % as calculated for *N. rustica*.

The alkaloidal composition of the 3 tobaccos was not modified qualitatively as a result of topping or grafting. Nicotine was the primary alkaloid in all cases. The Turkish and Connecticut Shade No. 49 varieties contained faint traces of nornicotine and anabasine. Topping the latter 2 varieties yielded somewhat more definite spots for nornicotine and anabasine in line with the general increase in alkaloid content as a result of topping. In general, *N. rustica* seemed to contain somewhat more of the secondary alkaloids than did the 2 varieties of *N. tabacum*. The minor alkaloids are not included in our quantitative data inasmuch as they are not distillable under our conditions of assay. A faint trace of nicotine was obtained in the tomato roots of grafted Turkish plants but the amount was too small to be measured by our routine assay procedure.

Discussion

The limitations and necessary assumptions of this work at various points above have been discussed in considerable detail. With respect to aerial parts, most of the limitations are in the direction of overestimate rather than underestimate.

In view of this situation, the data are all the more remarkable in indicating the extremely limited capacity for nicotine synthesis of the aerial organs of the tobacco plant as compared with the root system. Even when the plant is topped, no more than one-tenth of the nicotine content of the shoot, and usually much less, can be produced in the shoot. For reasons stated, the proportion is probably less than one-tenth. In the intact plant probably no more than 2 % and as little as 0.1 % of the nicotine content may be made in the shoot. The situation is made much clearer in the case of *N. rustica* by the virtual absence of transport of alkaloid from stem into leaves and from graft union into stems.

Of especial interest are the low figures for contribution of the leaf to its own nicotine content. The opinion that the leaf is a major site of nicotine production is still deeply rooted in the lore of tobacco cultivation. The weight of qualitative evidence against this idea has been increasing in recent years. The present work provides quantitative evidence of its incorrectness, and the results of the topping experiments indicate that there are not likely to be important exceptions.

These data cannot be further refined until means are found to estimate more accurately the amounts of alkaloids in the leaves, which are produced in the leaves.

Summary

The relative nicotine producing capacities in tobacco, shoot and leaf, have been estimated for intact and topped plants of 2 varieties of *N. tabacum* and 1 variety of *N. rustica*.

These estimates were based upon the assumption that the alkaloid contents of the stem and leaves of the grafted shoot, correspond to the intrinsic alkaloid producing capacities of these organs.

The limitations of this approach are discussed. It is pointed out that these limitations affect the estimates relative to nicotine production in shoot and in leaf of *N. tabacum* in the direction of overestimation as a result of some transport of alkaloid into stems and leaves of grafted plants from the graft union. This complication was not present in the case of *N. rustica*. In *N. rustica* about 0.10 % of the alkaloid contained in the shoot of the intact plant is produced in the shoot. Nearly all of this nicotine is located in the leaves. When plants of this species are topped,

these figures are not appreciably altered except that only about one-half of the total alkaloid of the shoot may occur in the leaf.

The 2 varieties of *N. tabacum* possess very similar nicotine producing capacities. In the intact plant, between 1 and 3 % of the alkaloid in the shoot was estimated to be produced in the shoot, but only about 0.5 % of that present in the leaf was estimated to be produced in the leaf. The effect of topping was to raise these figures to the level of 3 to 8 % and of 0.5 to 3 % respectively.

A partial correction of the data for Turkish tobacco indicates that substantially over 99 % of the total alkaloid in the plant is produced in the root system.

The authors are especially indebted to Mr. Joseph Stoeber of the Nevis Biological Field Station of Columbia University for growing the plants.

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A WIDE-RANGE A.C. BRIDGE FOR DETERMINING INJURY AND DEATH[1]

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Frequently in physiological and agronomical investigations it is necessary to know whether tissues are alive or dead or injured, so that a rapid method for making an appropriate diagnosis would be very useful. From the classical work of Osterhout (12) it is known that in water of high electrolyte content, the low frequency resistance of a tissue ultimately decreases with injury and falls to a low value on death. In such a medium, seriously injured or dead tissues are characterized by small values for low frequency resistance. Under other conditions, however, dead tissues are not similarly characterized. Thus in water of suitably low electrolyte content, increasing injury from a poison can result in an increasing low frequency resistance until at death a large and constant resistance value is obtained, as shown in fig 1 (for explanation see next section). In water of somewhat higher electrolyte content similar low frequency resistance values can be obtained in living and in dead sections (Greenham, unpublished). Moreover a dead and partially desiccated root can have the same low frequency resistance as a healthy root (5).

To overcome the difficulty caused by desiccation, measurements at both 1 kc/s and 100 kc/s were used (5), though 1 Mc/s would have been preferable for the higher frequency. More recently, to measure in-

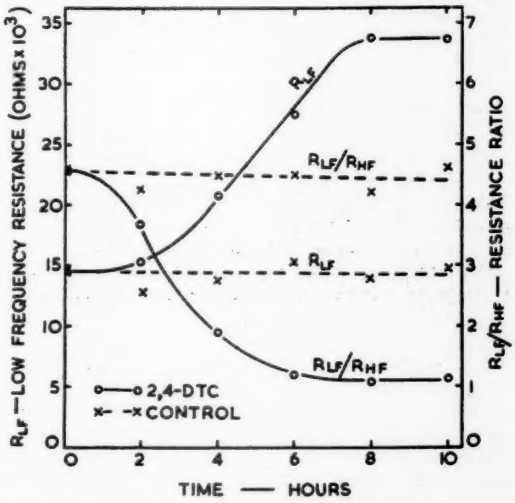

Fig. 1. Time-course of changes in electrical measurements on sections of etiolated pea stems. 2,4-DTC= 2×10^{-3} M 2,4-dichlorophenoxymethyl triethylammonium chloride; controls in distilled water. Sections equilibrated 20 hours beforehand in distilled water. Probe tips 2.5 mm apart, length exceeding diameter of sections. Measurements made with instrument described below.

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jury under conditions where low frequency resistance readings alone would be unsatisfactory, use has been made of the resistance ratio (resistance at 1 kc/s/resistance at 1 Mc/s, (4)) or the resistance index (resistance ratio × 100, (6)). The resistance ratio is comparatively high in healthy tissues (the value depending on the type of tissue and its pretreatment), may increase with initial injury, but decreases with further injury to give a value of approximately one at death ((4); see also below). This does not mean, however, that all tissues or sections with some other common resistance ratio, say 3.5, have been injured to the same extent. Any interpretation of the value of the resistance ratio, provided it is not approximately one, should be made in relation to the value for appropriate controls. So far it is known that injury resulting from poisons, cold, heat or drought can cause decreased resistance ratio values (4, 6) (also Greenham, unpublished). The ratio can be determined on a slice of tissue in an electrolyte solution with the aid of suitable electrodes (12) or on a plant in the field with the aid of probe tips. The former measurement need cause no injury whatever to the tissue. The slight wounding caused by probe tips may result in resistance changes with time, but does not prevent reproducible measurements from being made.

Formerly apparatus for high frequency measurements has been bulky, expensive and limited to laboratory use on account of both size and current consumption. This paper describes a portable and comparatively cheap wide-range A.C. bridge for determining impedances in terms of equivalent parallel resistance and capacitance at 1 kc/s, 10 kc/s and 1 Mc/s. The intermediate frequency is one which has been used for discriminating between healthy and virus-infected potato tubers (8). The instrument not only has been used for all measurements of the types described in the above references (within the limits given in table 1), but also has been used for measurements of soil moisture in terms of resistance (2) and capacitance (1, 3); moreover, it is suitable for resistance measurements on plants growing in soil.

Although previous experience in the use of A.C. bridges is unnecessary for operating the instrument described below, a knowledge of radio practice is essential for its construction.

Discussion

The electric circuit representing a healthy tissue contains a number of elements of resistance and capacitance and accordingly comprises an impedance. Often when electric measurements are made on a tissue, its impedance is matched or balanced against an equivalent parallel resistance and capacitance in an A.C. bridge; at balance the phase angle and impedance of the tissue respectively equal those of the equivalent parallel resistance and capacitance. While it is possible to calculate the impedance and phase angle of the tissue from the equivalent parallel resistance and capacitance it is often adequate to determine the physiological condition of the tissue solely in terms of the magnitude of the equivalent parallel resistance (frequently called resistance by biologists).

The reciprocal of equivalent parallel resistance, viz. equivalent parallel conductance (conductance or conductivity), is used in some biological investigations, e.g. (7). However, the term conductance should not be confused with the reciprocal of impedance, known as admittance.

A capacitor (condenser) opposes the flow of electricity caused by low frequency alternating currents, but this opposition becomes less as the frequency is increased and can become negligible at high frequency. Thus a combination of capacitances and resistances, as occurs in healthy tissues, has a high impedance at low frequency (e.g. 1 kc/s) but a low impedance at high frequency (e.g. 1 Mc/s). Luyet (11) has shown experimentally that the high frequency resistance of a living tissue is approximately the same as the low frequency resistance of that tissue when dead (provided desiccation or change in electrolyte content has not occurred). When a tissue is dead it has no capacitance, so its resistance at 1 kc/s is equal to its resistance at 1 Mc/s, no matter whether the tissue is wet or dry[2]. The magnitude of the ratio of the low frequency resistance of a tissue to its high frequency resistance frequently gives a useful measure of the condition of the tissue, provided the tissue is not both dormant and dry (seeds, lichens, etc.), or wilted.

Dead tissues can have high electric resistance at any frequency if a) the tissues are dry, b) if the tissues have been left in water low in electrolyte content. In both instances the high resistance is due to low ionic conduction, in a) to the low water content, in b) to low electrolyte content, most of the electrolytes having leached into the water. On the other hand, the high resistance of a living tissue at low frequency is attributed classically to the plasmalemma or cell membrane (12). The decrease in low frequency resistance caused by injury not accompanied by loss of water and electrolyte is attributed mainly to increased ionic conduction across the plasmalemma. With injury in very dilute solutions, however, the ability for increased ionic conduction across the plasmalemma may be more than offset by the loss of ions to the external medium, and the low frequency resistance of a tissue may accordingly rise (fig 1).

Although the existence of the plasmalemma has been questioned (9), it is considered real in view of direct current measurements (13).

[2] If metallic probes are used, the reactance at their surface (5) will affect measurements at low frequency far more than those at high frequency. Accordingly the resistance ratio may be 1.2 or 1.1 in dead wet tissues, about 1.6 in dead dry tissues (5 fig 9). Due allowance can be made for these increased ratio values when interpreting results.

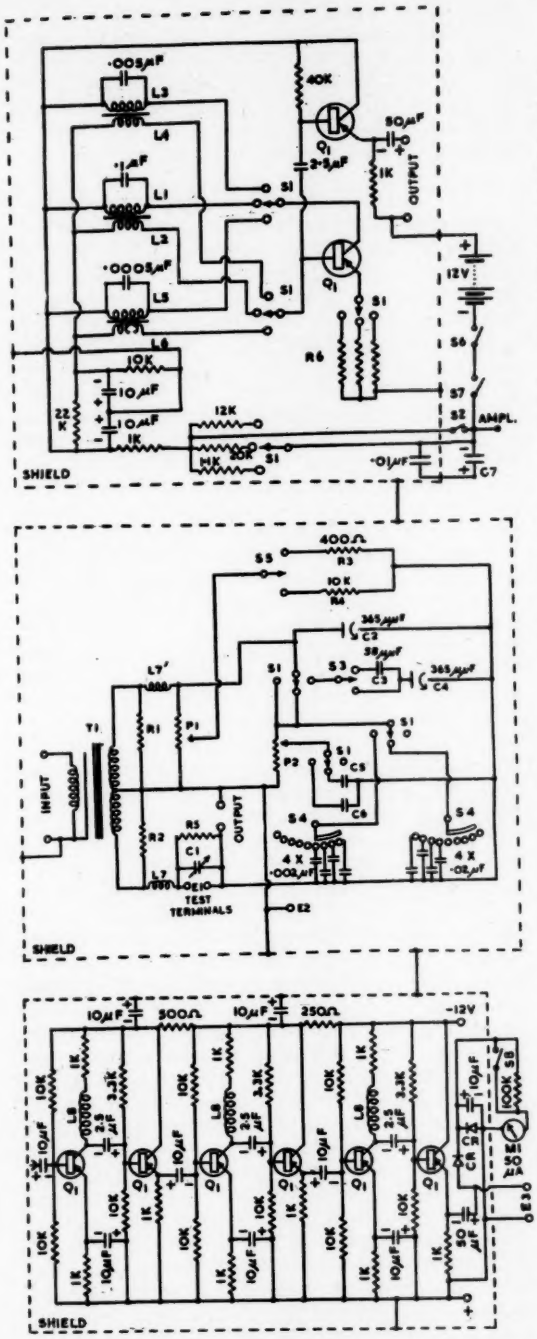

FIG. 2. Circuit for wide-range A.C. bridge. (*top*), Oscillator with isolation stage. (*center*), Bridge transformer and bridge unit. (*bottom*), Amplifier, rectifier and meter.

MATERIALS

The design (fig 2), is such as to minimize errors when grounded objects, e.g. plants in soil, are being measured. The design, moreover, includes a number of approximations which facilitate construction and portability but which leave sufficient accuracy for many biological measurements. For convenience, the one type of transistor has been used throughout. The apparatus consists of four basic portions: a) oscillator with isolation stage, b) bridge transformer with bridge unit, c) amplifier unit and rectifier and d) a meter as balance detector. Further description is limited mainly to portion b) which includes the more novel features of design.

Conventionally, metal strips are used for the secondary windings of bridge transformers (10). However, by including correcting resistors R 1 and R 2, mentioned later, it is possible to use wire for the secondary windings of T 1 (see appendix I).

The bridge unit measures impedance in terms of resistance and capacitance in parallel. As in some commercial instruments, variations in resistance are simulated by a potentiometer, P 1, though two limiting resistors are used. R 3 allows a useful range for P 1 of 400 to 10,000 ohms, and R 4 a useful range of 10,000 to 500,000 ohms. It is convenient to calibrate the outer portion of the scale of P 1 for the lower range, and the inner portion for the higher range.

Two separate units are used for capacitance measurement, the appropriate unit being automatically selected by S 1. At 1 Mc/s C 4 is used, its maximum of 365 $\mu\mu$F being reduced to 50 $\mu\mu$F by C 3 and S 3; the lower value facilitates bridge balance under some conditions but does not need a separate scale. At 1 kc/s and 10 kc/s potentiometric division of C 5 and C 6 is used (3), permitting maximum values of 0.02 μF and 0.002 μF respectively without serious phase angle errors. Supplementary capacitors may

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0.002 μF, 1 % (see table I). C 7, 750 μF 12 V.P. electrolytic capacitor. CR Germanium diode.

L 1-L 6, see Appendix I. L 7, L 7', see text (Adjustment, calibration and use). L 8, see Appendix I.

P 1, 1,000 ohm Ayrton-Perry wound potentiometer or 1,000 ohm precision carbon potentiometer. P 2, 100 ohm Ayrton-Perry wound potentiometer (obtained from British Electric Resistance Co., Queensway, England).

Q 1, Raytheon 2 N 112/C K 722 transistor.

R 1, R 2, see text (Adjustment, calibration and use). R 5, 1.5 Megohm 1 W high stability non-reactive resistor. R 6, approximately 2.5 K each. Adjust for best waveform. S 1, Main selector switch (frequency, capacitance measuring controls and dropping resistors for battery). Wafer type, 2 bank, 4 pole, 3 position. Note: In fig. 2, the top or left-hand contact is used at 10 kc/s, the center contact for 1 kc/s and the remaining contact for 1 Mc/s. S 2, press OFF switch for low sensitivity (spring return). S 6, manual ON/OFF switch. S 7, Automatic ON/OFF switch (operated by lid of case). S 8, press ON switch for high sensitivity (spring return).

T 1, bridge transformer (see Appendix I).

be introduced by S 4 to give an upper limit of 0.1 μF at 1 kc/s and 0.01 μF at 10 kc/s.

High quality components should be used particularly in the bridge unit. Moreover, in this unit all resistors must be non-reactive and of high stability. As high stability 100 ohm carbon potentiometers have not been available, an Ayrton-Perry wire wound potentiometer has been used for P 2. If such a potentiometer is used for P 2, however, it is disconnected from the circuit at 1 Mc/s, otherwise the calibration of P 1 at that frequency is no longer within the limits specified in table I. The differences in the loading of T 1 at 1 Mc/s and lower frequencies cause no trouble if R 1 and R 2 are adjusted as described later and T 1 is made to specifications.

Compensating inductors L 7 or L 7′ may or may not be required, depending on the length of the leads and the difference in inductance between the two bridge arms. C 2 is provided to balance the two arms before making measurements. R 5 is included to ensure that the scale of P 1 can extend to infinite resistance at 1 kc/s. Adjustments are described in the next section.

To facilitate preliminary balance of the bridge, S 2 is included to reduce the output of the oscillator as required. S 8 subsequently permits more accurate balancing of the bridge.

It is impossible to over-emphasize the need for good shielding between portions a), b) and c); shielding also applies to the wiring between these portions. A metal sub-panel, beneath the top insulating panel, is used as part of the shielding. The case, if made of metal, is insulated from the sub-panel by at least 0.5 cm clearance, and thereby lessens errors due to ground capacitance. In normal use the shield of the instrument is not grounded.

Total current consumption is 34 ma. at 12 volts, so for long life dry cells at least 6 cm × 3.5 cm dia. should be used for the battery.

The prototype instrument is housed in a metal case 30 cm × 26 cm × 14 cm, and weighs 6.4 kg.

Methods

If capacitance measurements are to be made, C 2 is provided with a scale so that its appropriate balance position can be determined beforehand for each frequency and each resistance range. The balance position of C 2 is the position required to give zero meter deflection with P 1 set at infinite resistance, P 2 or C 4 set at zero capacitance, and with infinite resistance (other than R 5) and zero capacitance (other than C 1 and the capacitance of the leads) across the test terminals.

Even if capacitance measurements are not required, at 1 Mc/s it is desirable to use for the leads a constant length of twin flex or preferably a constant length of shielded cable with shield connected to E 2, because the use of leads of a different length or of a different construction can cause errors in the resistance measurements. The total length of leads for measurements at 1 Mc/s should not exceed say 70 cm, but is not so critical at the lower frequencies.

It is preferable to connect a cathode ray oscilloscope across terminals E 3 so that the following adjustments at high values of resistance can be carried out with suitable precision. Once the instrument is calibrated, however, the meter should be sufficiently sensitive for measurements on tissues and soils.

Adjustment of the Bridge is as follows: a 10,000 ohm carbon potentiometer, referred to below as P 3, is substituted for resistors R 1 and R 2 and the moving contact is connected to the center tap of the transformer. The compensating inductance is included in the circuit in the position shown for L 7, and is a self-supporting coil of 14 American wire gage enamelled wire, consisting initially of about five turns of say ¾ inch diameter. A carbon or other non-reactive resistor of 40,000 ohms is connected to the test terminals by means of the leads or cable selected for subsequent use. The moving contact of P 3 is set at its center position, switch S 1 at 1 kc/s, and S 3 to the 365 μμF range. P 2 is turned to zero, S 5 is set at the upper resistance range, and P 1 and C 2 are adjusted until the bridge is balanced. S 1 is now set at 1 Mc/s, C 4 is set at zero, and C 2 and P 3 are adjusted several times until the bridge is balanced, P 1 not being altered.

The 40,000 ohm resistor is now replaced by a 10,000 ohm non-reactive resistor, and S 5 is moved to the lower resistance range. C 4 is set at about 30 μμF and C 2 and P 1 are adjusted until the bridge is balanced. A 400 ohm non-reactive resistor is now substituted for the other, and P 1 and C 4 are adjusted for bridge balance, P 3 and C 2 not being altered. If the capacitance of C 4 is now more than before, the inductance of L 7 is not enough, whereas if the capacitance is less than before, the inductance is too high or should be in the position shown for L 7′. The inductor should be altered as necessary, and the whole adjustment procedure for the bridge is repeated, starting with the setting of the frequency switch S 1 at 1 kc/s, mentioned in the previous paragraph.

After several such adjustments of the bridge an adjustment of the correcting inductor should be obtained such that on changing P 1 from the 10,000 ohm setting to the 400 ohm setting, the capacitance of C 4 does not need to be altered to obtain bridge balance. P 3 is carefully removed from the apparatus and the resistance values on either side of the moving contact are measured. Fixed high stability non-inductive resistors of the values determined are then soldered in place of P 3.

Scale calibration of P 1 is done at 1 kc/s, but before this is done P 2 should be set at its zero position and C 2 set at its balance position. Scale calibration of P 2 is also done at 1 kc/s, with infinite resistance (other than R 5) in both arms. The graduations of P 2 should be satisfactory for use at 10 kc/s, although the calibration at this frequency will be one-tenth of that at 1 kc/s. Capacitance C 4 can be calibrated only at 1 Mc/s, with infinite re-

TABLE I.

ACCURACY, WIDE-RANGE A.C. BRIDGE

RESISTANCE RANGE	CAPACITANCE RANGE	FREQUENCY	RESISTANCE ERRORS	CAPACITANCE ERRORS
Ohms				
400–10,000	0–0.1 μF	1 kc/s	1–2 %	1–2 %*
10,000–100,000	0–0 02 μF	1 kc/s	1–2 %	1–2 %
100,000–500,000	0–0 02 μF	1 kc/s	2–5 %	1–2 %
400–10,000	0–0 01 μF	10 kc/s	1–2 %	1–2 %
10,000–40,000	0–0 002 μF	10 kc/s	1–2 %	1–2 %
40,000–80,000	0–0 0005 μF	10 kc/s	2–3 %	2 %
400–10,000	0–200 μμF	1 Mc/s	1–2 %	0.5–2 μμF
10,000–40,000	0–50 μμF	1 Mc/s	2–3 %	0.5–1 μμF

* Percentage values refer to at least half-scale rotation of P 2. Errors may be greater at less rotation due to errors in scale reading.

It is assumed that C 6 is 1/10 of C 5 to within 0.5 %. Supplementary capacitors introduced by S 4 should be correct to within 1 %.

sistance in both bridge arms and with C 2 set at its appropriate balance position.

Table I shows the percentage error in the calibration of the prototype instrument. These errors are not important in many biological or agricultural measurements where variation causes greater errors and where there may also be slight injury caused by the probe tips. Table I also shows the upper limits where the stated errors apply. Such limits are considered reasonable for measurements on most tissues which would have to be partially desiccated or low in electrolyte content to give equivalent parallel resistance values of 80,000 ohms at 10 kc/s or 40,000 ohms at 1 Mc/s.

The meter is now ready for use. The unknown is connected to the leads or cable, the appropriate frequency is selected, and capacitor C 2 is set to its appropriate balance position if capacitance readings are required. Controls P 1 and P 2 or P 1 and C 4 (depending on frequency) are adjusted for balance, as described in Appendix III. With experience, and provided abnormally high capacitance values are not encountered, it should be possible to make a reading at one frequency within 20 seconds.

For accurate measurements at 1 Mc/s it is necessary to minimize errors caused by resistance or capacitance to ground (these errors are negligible at the lower frequencies). Thus, if detached apples or other objects are being measured in the laboratory and it is necessary to hold such objects, a rubber glove should be used to insulate the object from the hand. To avoid holding an object, it can often be impaled on vertical probe tips. Readings can be made satisfactorily on a plant in a small pot on an insulated table. Resistance measurements at 1 Mc/s on plants in the field may be less accurate due to the test objects being grounded, but the errors are usually of the order of 2 to 5 pc, and are negligible for field determinations. Capacitance readings in the field at 1 Mc/s may have larger errors, but are seldom required. Because the test object is being grounded, however, the meter reading may not be zero at balance.

SUMMARY

Details are given of a portable A.C. bridge operating at 1 kc/s, 10 kc/s and 1 Mc/s, and capable of measuring impedances in terms of equivalent parallel lumped resistance and capacitance. It can be used for diagnosing whether tissues are alive or dead or injured, for conductance measurements, and for the measurement of soil moisture in terms of both resistance and capacitance.

APPENDIX I. CONSTRUCTION OF BRIDGE TRANSFORMER AND OSCILLATOR COILS.

TRANSFORMER: The core, manufactured by N. V. Philips Gloeilampenfabriekan, Eindhoven, Holland, is marketed as Ferroxcube. Core specifications are: Type No. 56 907 18/3 A. Size (single E section), length 52 mm, height 30 mm, thickness 12 mm. Initial permeability of core material 3 A at 20° C, 1,400. Two E sections are used.

Primary winding consists of 240 turns of silk-enamel Litz wire (27 strands of 0.002 inch dia.), in 4 layers, with leads at opposite side of transformer to secondary leads. The end of the top layer is connected to the general shield.

Electrostatic shield, between primary and secondary, consists of 1¼ turns of open insulated 0.002 inch shim brass (this shield must remain open).

SECONDARY WINDINGS: These consist of 2 lengths of 16 or 17 A.W.G. enamel wire, each of 10 turns, bifilar wound as a single layer. On completion the start of one winding is joined to the finish of the other, the junction forming the center tap. The two remaining leads become the outer connections for the two windings.

The completed transformer is enclosed in a can, the leads being brought out with as much spacing as possible between primary and secondary.

In the completed bridge the effect of reversing the connections to the secondary windings should be tried. Sometimes better results are obtained on reversal.

OSCILLATOR COIL: *Core for* 1 *kc/s coil assembly* (L 1-L 2): 2 Philips Ferroxcube E sections type No. 56 907 36/3 A. Size each E section 41 × 22 × 9 mm (900 turns required for 1 henry). L 1 is 350 turns 29 A.W.G. enameled wire. L 2 is 20 turns 29 A.W.G. enameled wire.

Core for 10 *kc/s coil assembly* (L 3-L 4) *as above*: L 3 is 175 turns 29 A.W.G. enameled wire. L 4 is 10 turns 29 A.W.G. enameled wire.

Core for 1 *Mc/s coil assembly* (L 5-L 6): 1 Philips Ferroxcube pot core type D 25/16 material 3 B1. L 5 is 15 turns 27/0.002 inch Litz wire. L 6 is 4 turns 27/0.002 inch Litz wire.

L 8 is Peaking coils (3) medium Q, adjusted to resonate near 1 Mc/s to improve amplifier response at 1 Mc/s.

Note: that the above Litz wire is also specified as 27/47 British standard wire gage.

Appendix II. Test and Probe Data

Transformer Input: 1 kc/s, 0.25 v RMS; 10 kc/s, 0.15 v RMS; 1 Mc/s, 0.8 v RMS.

Voltage RMS across test terminals, not exceeding 8 % of above values. Resulting current density through a 3 mm dia or larger tissue slice in an electrolyte solution is not injurious. With probe tips as specified below, wound injury, though slight, is more important than electrical injury is.

Amplifier Gain (Voltage): 1 kc/s and 10 kc/s, 2.7×10^4; 1 Mc/s, between 3×10^3 and 6×10^3. (Value at 1 Mc/s could not be measured accurately).

Probes: These are made from the tips of nickel-plated sewing needles about 0.5 mm in diameter. Length of probe tip depends on depth of penetration required; tips 1.5 to 3.5 mm long have been used. Separation of tips depends on test objects, but is conveniently 2.5 to 5 mm.

In use the probe tips must be free from grease or rust.

Appendix III. Procedure for Balancing Bridge

The following account is given for those not conversant with A.C. bridges. In the above instrument there are three stages of sensitivity, viz., low (with switch S 2 depressed), normal and high (with switch S 8 depressed). Until sufficient experience has been obtained it is preferable to start with the low sensitivity range and the lowest frequency required, also with switch S 5 on the low resistance range.

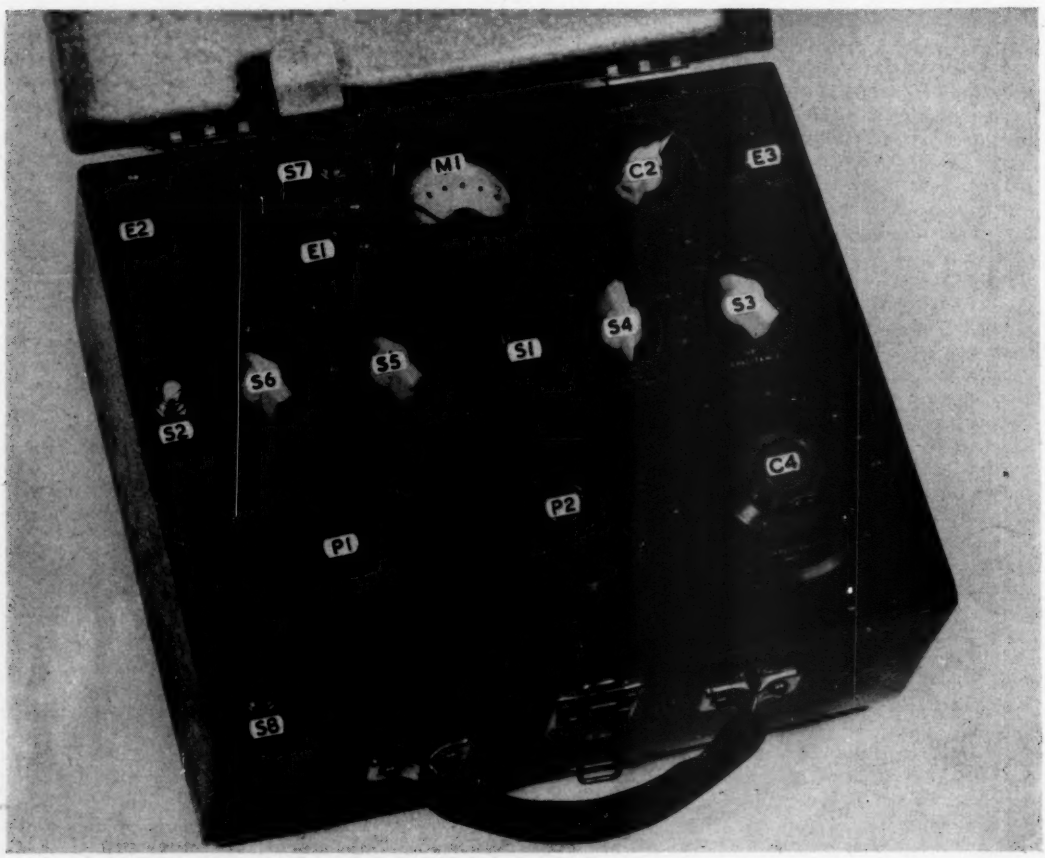

Fig. 3. Wide range A.C. bridge.

With S 2 depressed, C 2 at the appropriate balance position and P 2 and C 4 at zero capacitance, the resistance dial (P 1) is adjusted so that the meter deflection is a minimum, then the appropriate capacitance dial (C 4 at 1 Mc/s, P 2 at lower frequencies) is adjusted until the meter deflection is a further minimum but preferably zero. S 2 is released, and both dials are again adjusted until the meter deflection is a minimum but preferably zero. If a more accurate setting of the dials is required, S 8 is depressed and the dials are again adjusted so that the meter deflection is a minimum or preferably zero. If the resistance reading is more than 10,000 ohms, a more accurate reading can be obtained on the high resistance range.

At final balance the dial reading of P 1 or P 2 or of both is taken, the reading of C 4 seldom being required. In most physiological investigations only the resistance reading is needed.

It should be noted that:

a) For an accurate balance of the bridge several adjustments of P 1 and of P 2 or C 4 may be necessary, because the adjustment of one dial can affect the subsequent setting of the other.

b) If, with S 2 depressed for low sensitivity, the dial of P 2 has to be turned to its maximum position for minimum meter deflection (which is not zero), it may be necessary to use switch S 4 to introduce extra capacitance and then to readjust P 2 to obtain zero meter deflection. The subsidiary capacitors, however, will normally be needed only for severely injured or dead wet tissues.

c) With the instrument at the normal sensitivity range, an increase in meter deflection may precede a decrease, as a result of non-linearity of the amplifier due to overloading.

d) The high sensitivity range should be used only when the meter deflection on the normal range is at 2 microamperes or less.

e) Particularly on the high sensitivity range, zero meter deflection cannot always be obtained at "balance" because the potentiometer wiper arms cannot resolve less than one turn of wire.

Lack of zero meter deflection at balance can be due to 4 other causes, viz., insufficient shielding between functional units, harmonic content of the input to the bridge transformer, insufficient decoupling between the oscillator and amplifier units, and the use of twin flex for the leads instead of shielded twin cable. These troubles should not occur in an instrument constructed as described.

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NITROGEN MOBILIZATION IN PEA SEEDLINGS [1, 2]

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The processes by which the storage proteins of seeds, especially legume seeds, are converted to the proteins of the growing organs of the seedling have been an object of study since the early days of plant biochemistry (4). It has usually been believed that the reserve proteins are hydrolyzed to their constituent amino acids, which are interconverted before or after their transport to the site of new protein synthesis. The interconversions lead to the proper array of amino acids to make up the new proteins. However, recent evidence that many of the amino acids in carrot root tissue cells are not in equilibrium with the precursors of newly synthesized proteins (22), and observations on the close relationship between seedling respiration and protein metabolism (25) have led to the hypothesis of a "protein cycle" in which proteins are considered to form a substrate for respiration and CO_2 production, while new proteins are formed from incorporation of ammonia into carbon skeletons derived from carbohydrate. It would seem likely that plants use both mechanisms, and use them to different degrees in different organs, growth stages and species. Whether the amides play a different or more important role in these transformations is also not clear, although it seems well established that asparagine can serve as a repository for excessive exogenous or endogenous supplies of ammonia (17).

Goksøyr et al (10) have presented data indicating that in the pea seedling there is actually no early mobilization of nitrogen from the reserve protein until the non-protein nitrogen of the cotyledons is depleted; a similar statement was made in a more recent report (21). The more detailed experiments of Danielsson (8) indicate that the reserve proteins, particularly the globulins, decrease slowly at first and then more rapidly when the root-shoot axis begins to show rapid growth. Up to this point dialyzable nitrogen in the cotyledon was increasing rather than decreasing.

It seems clear that many questions about the early life processes of the seedling remain unsettled. We have chosen the young pea seedling for study since it grows to considerable size while using only the food supplies stored in the seed. Thus complications of processes of nitrogen assimilation and photosynthesis are avoided. Previous studies on peas have been on the seedling as a whole, or in a few cases, on cotyledons and root-shoot axis. The present experiments treat the root and shoot tips separately. This report provides a picture of the broader aspects of the changing nitrogenous constitution of the different types of seedling tissue. Study of the more detailed changes is continuing.

MATERIALS

Pea seeds (*Pisum sativum* L. var. Unica) (11) were obtained from Holland. The peas were surface sterilized by immersing them in 0.5 % sodium hypochlorite for 30 minutes, followed by 2 washings with sterile water. They were planted 2 inches deep in uniformly washed vermiculite (plaster aggregate) supplied with distilled water with no added nutrients. Growth was in an incubator at 18° C, and samples were taken at 1, 3 and 5 days, by which time the seedlings had not emerged through the surface. At 1 day, all seeds, except the few discolored ones, were used for the samples. Older seedlings were selected for uniformity by using, at 3 days, those between 35 and 70 mm long from root tip to shoot hook, and at 5 days, those between 115 and 180 mm long (usually about 80 % for both).

After washing the seedlings free from vermiculite, the seed coat was removed. For the 1 day samples, the embryonic root-shoot axis (including the cotyledon petioles) was dissected from the cotyledons. The 3 and 5 day samples were dissected into five parts: the cotyledons; the shoot tip, to the middle of the hook; the shoot shaft, including the cotyledon petioles; the root tip, about 2.5 mm long; and the root shaft. All parts were immediately frozen on dry ice, and kept frozen until lyophilized; then they were ground in a mortar (or in a micro-Wiley mill for cotyledons) and stored at −18° C until analyzed. Where necessary, lyophilization was continued for longer than overnight in order to bring the samples to less than 5 % moisture. No correction for this residual moisture has been made in the calculations, but mechanical losses during grinding were allowed for.

The initial seed samples were dissected without any preliminary softening by moistening. In fact it was found best to reduce the normal moisture content by drying the seeds in a vacuum desiccator for about a week. Each seed then cracked readily when struck lightly with a pestle, the seed coat fell away, and the embryonic root-shoot axis was cut away from the cotyledons mainly on the basis of its different color.

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[2] Scientific Paper No. 1848, Washington Agricultural Experiment Stations, Pullman, Project No. 769.

FIG 1. Diagram of 3-day seedling. The dotted lines show the point of dissection of root tips and shoot tips. The rectangles indicate the approximate position from which the sections shown in figures 2 and 3 were taken.

The root and shoot tips were cut off as indicated in figure 1 at points chosen on the basis of sections such as those shown in figures 2 and 3 so as to roughly separate the meristematic cells of the tips from the older non-dividing cells.

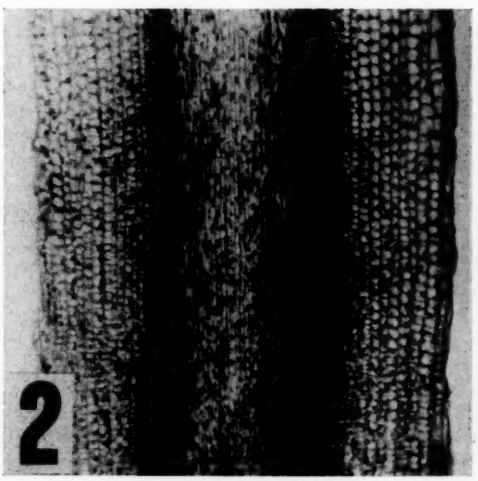
METHODS

Total nitrogen was determined on a weighed portion of the whole ground sample. Alcoholic extracts were analyzed for total nitrogen, amino nitrogen, amide nitrogen, ammonia nitrogen, urea nitrogen and peptide nitrogen. Peptides were also determined on the residues from the alcoholic extractions. Protein nitrogen was considered to be represented by the remainder after subtraction of the alcohol-soluble nitrogen and alcohol-insoluble peptide nitrogen from the total nitrogen. All water used was redistilled from glass before use.

Work with an earlier set of samples similarly prepared had shown that the nitrogen fractions of the seed coat were low in amount and changed little during seedling development, so they were not included in the analyses.

TOTAL NITROGEN: Cotyledons, root shaft and shoot shaft samples were analyzed for total nitrogen by the macro-Kjeldahl method. The micro-Kjeldahl method (*2*), modified by the use of Nessler's solution on the distillate rather than titration, was used for all other types of samples. No appreciable nitrate was found in pea seeds.

ALCOHOLIC EXTRACTION: The samples were extracted by homogenizing 400 mg (5 g of cotyledon



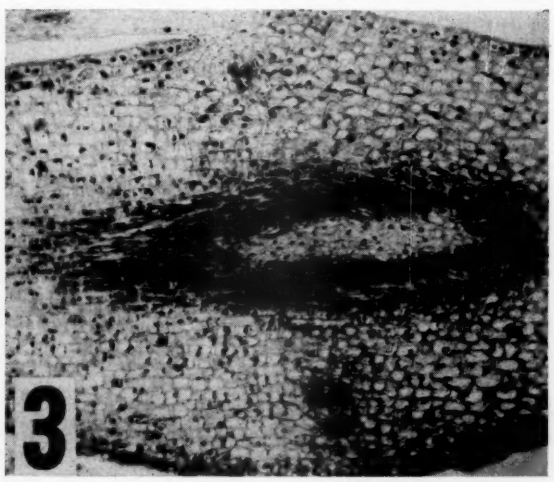


FIG 2. Longitudinal section through root of 3-day seedlings. Dissection was at a point near the center of the figure, 2.5 mm from the tip.

FIG 3. Section through hook of 3-day pea seedling, tangential to the stele. Dissection was a point near the center of the figure.

samples) of ground sample with 8 ml (60 ml for cotyledons) of a 70 % (v/v) ethanol solution and vigorously shaking the suspension at room temperature for at least 6 hours. The suspensions were then centrifuged, supernatant solutions were decanted, the residues were well mixed with another portion of 70 % alcohol, and the shaking and centrifugation were repeated. The extractions were repeated twice more; the residues were washed briefly with another small volume of alcohol; the extracts and washings for each sample were combined and clarified by briefly centrifuging at about 12,000 × G, and were made up to a volume of 50 ml (300 ml for cotyledons). The extracts were stored at −18° C. until analyzed. The residues were partially dried at room temperature, then in the vacuum oven, and also stored at −18° C.

If further extractions are made, only very small amounts of nitrogen are still obtained in the extracts. The experiment reported in table I shows that 4 extractions and a washing remove at least 93 % of the nitrogenous material obtained in 6 extractions. The nitrogenous material in the later extractions may represent partly the equilibrium solubility of largely insoluble material, or it may be insoluble material whose solubility has been changed, or it may be insoluble material in fine suspension, as evidenced by the slight turbidity before clarification in the angle centrifuge.

Alcohol-insoluble Peptides: The method is based on that of Bathurst (3). The dried residue from the alcoholic extraction was shaken for 2 hours with 10 ml of water (80 ml for cotyledon samples); centrifuged; the supernatant extract was decanted; the residue was washed once with water, and the washings and extract were combined. A few drops of glacial acetic acid were added until no more precipitation resulted. The pH at this point was about 4.0 to 4.5. The precipitate was centrifuged down, and the supernatant solution was made up to 25 ml volume (100 ml for cotyledons). Aliquots of this solution were taken for submicro-Kjeldahl nitrogen determination with sealed tube digestion (2) and distillation by the Conway microdiffusion method (6) using 0.6 ml of 40 % KOH as the alkalinizing agent, 2 ml of 0.002 *N* HCl as the absorbing agent, diffusing overnight at room temperature, and back-titrating the excess acid with 0.02 *N* $Ba(OH)_2$.

The identification of this as a peptide fraction is, of course, rather arbitrary. With cotyledons, it was shown experimentally that the acetic acid treatment precipitated as much nitrogen from the water extract of the residue from the alcoholic extraction as did 3 % trichloracetic acid, which is believed to precipitate proteins but not protein intermediate products (12).

Table I

Cumulative Percentages of Nitrogenous Substances Extracted by Repeated Treatments with 70 % Ethanol

No. of extractions	From cotyledons of 3-day seedlings		From shoot shafts of 3-day seedlings	
	Total N	Amino N	Total N	Amino N
1	51	55	66	64
2	76	82	91	86
3	86	91	97	98
4	91	95	98.7	99.3
4 + washing	93	97	99.0	99.7
4 + wash + 1	96	98	99.5	99.9
*4 + wash + 2	100	100	100	100

* Assumed to extract 100 %.

Alcohol-Soluble Nitrogen: The total nitrogen content of the alcoholic extracts was determined by the micro-Kjeldahl method (2).

Free Amino-Nitrogen: The method of Rosen (19) was used. All the common α-amino acids, including asparagine gave approximately equal molar color yields, as did also γ-amino butyric acid. β-Alanine yielded only 30 % as much color, and proline, hydroxyproline and pipecolic acid gave little or no color at 560 mμ. On the other hand free ammonia gives about 60 % molar color yield. However, very little ammonia was found, and on balance, one would expect the method to give a good estimation of the true amino nitrogen content.

Free Ammonia Nitrogen: The ammonia level in the samples was so low that it was necessary to carry out preliminary 2-fold to 5-fold concentrations by blowing hot air on the surface after first adjusting the pH to about 5.5. Ammonia was then determined by the Conway microdiffusion method without preliminary digestion, with 1 ml of saturated borate (1) as the alkalinizing reagent, 1 ml of 0.002 *N* HCl as the absorbing acid, diffusing 2 hours at 37° C. and titrating the excess acid with 0.02 *N* $Ba(OH)_2$. This method gave an average recovery of ammonia from $(NH_4)_2SO_4$ of 98 %, while releasing only about 2 % ammonia from glutamine. The ammonia levels were so low, however, that the overall precision of the analyses was rather poor.

Amide Nitrogen: Amide nitrogen plus preformed ammonia was determined by the modified method of Borsook and Dubnoff (6). The alcoholic extracts were concentrated (see above) before analysis. Following the digestion, the ammonia was determined by micro diffusion as described above for peptides. Under the conditions of this procedure, urea released about 20 % of its nitrogen as ammonia. This amount caused no significant error in the light of the levels of urea found to be present. Two series of recovery experiments with asparagine (the more stable amide) yielded average recoveries of 104 and 96 %.

Urea Nitrogen (6): One hour's incubation with urease at room temperature was used, followed by de-

termination of the ammonia released as in the method for free ammonia previously described. The alcoholic extracts were concentrated (see above) before analysis. Recovery experiments with urea indicated a loss of about 10% during the concentration step, and another 5 to 8 % loss in the analysis. Results reported are uncorrected, except for subtraction of the preformed ammonia.

ALCOHOL-SOLUBLE PEPTIDES: The method of Markovitz and Steinberg (15) was used. Great pains had to be taken to exclude ammonia during the process, at the level of peptide nitrogen involved. Even so, reproducibility of analytical results was not good, and the data reported for soluble peptides have a relatively low precision.

RESULTS AND DISCUSSION

The analytical results are summarized in tables II and III. Each of the figures reported represents the average for 3 to 5 replicates. Each of the replicates was made up of 200 to 300 plants, in the case of the 0- and 1-day samples, and 1,000 to 2,000 plants in the case of the 3- and 5-day samples. (In the latter cases more were required because individual root tip samples were so small.)

(It would not be practical to report statistical parameters for all cases in these tables, but a few examples should give a reasonable basis for judging the significance of trends. The standard deviation of the mean of 4 replicates for alcohol-soluble nitrogen in the cotyledons was 4.1 parts per hundred; for the amino nitrogen in cotyledons, 4.7 parts per hundred, and for amide nitrogen in cotyledons, 6.7 parts per hundred.)

Though the results do not disprove the participation of a protein cycle, they fit in well with the simpler theory of more direct conversion. The picture revealed in table II is of an accelerating consumption of cotyledon protein paralleling an accelerating growth rate of the growing parts, as indicated by dry weight. The protein of the growing parts increased steadily. The alcohol-soluble forms of nitrogen, presumably intermediate between reserve protein and new protein, in the cotyledon rose rapidly at first, but were falling by the 5th day; while in the growing parts, these forms rose slowly at first and then at an increasing rate. This was true of amino nitrogen, amides and smaller peptides. By the 5th day, there was more alcohol-soluble nitrogen in the growing tissues than in the cotyledons.

Results of an earlier unpublished study suggested that the same pattern holds at other growth rates. This was the same sort of study as the present one, using Alaska peas, and growing them in vermiculite washed with benzene and petroleum ether as well as water. It was found that benzene left a residue which reduced the growth rate of the seedlings. Nevertheless, a very similar pattern prevailed; a 4-

TABLE II

NITROGENOUS CONSTITUENTS OF PEA SEEDLINGS *

TYPE OF TISSUE	LYOPHILIZED WEIGHT	TOTAL N	PROTEIN N	ALCOHOL-INSOLUBLE PEPTIDE N	ALCOHOL-SOLUBLE N	FREE AMINO N	AMIDE N	AMMONIA N	UREA N	ALCOHOL-SOLUBLE PEPTIDE N
Pea seeds										
Cotyledons	21100	930	810	54	64	19	6.5	1.2	0.4	6.0
Root-shoot axis	250	17	14	1.2	1.9	0.47	0.09	0.04	0	...
Seed coat	2080	15	...	...	1.3	0.34	...	...	...	...
One-day-old seedlings										
Cotyledons	21900	944	802	48	94	23	8.6	0.5	0.9	9.0
Root-shoot axis	305	22	17.5	1.2	3.3	0.92	0.19	0.03	0.04	...
Three-day-old seedlings										
Cotyledons	21000	886	740	29	118	42	9.0	2.0	0.8	15
Shoot tips	133	11	8	0.2	2.9	1.1	0.28	0.1	0.03	0.1
Shoot shaft	580	35	18	1.1	16	8.8	1.5	0.5	0.3	0.8
Root shaft	500	31	13	0.7	17.5	9.9	1.6	0.5	0.3	0.6
Root tips	43	3.8	2.9	0.05	0.8	0.4	0.05	0.02	0.01	...
Five-day-old seedlings										
Cotyledons	17400	750	614	34	102	36	6.0	1.3	0.4	13
Shoot tips	140	12.5	9.5	0.2	2.8	1.0	0.44	0.1	0.05	0.2
Shoot shafts	1415	88	31	1.8	55	33	6.0	1.2	0.7	1.6
Root shafts	900	57	23	1.5	33	20	2.7	0.9	0.5	1.4
Root tips	32	2.5	1.8	0.05	0.6	0.3	0.03	0.02	0.01	...

* Mg per 100 seedlings.

day seedling in the earlier study approximated a 3-day seedling in the present work.

In these experiments the reserve protein of the cotyledons served as the source of nitrogen from the beginning. In agreement with Danielsson (8), the cotyledon non-protein nitrogen was observed to rise during the early stages of germination rather than to be consumed as reported by Goksøyr et al (10).

At first the amides accounted for about one-third of the free amino nitrogen of the cotyledons, and a smaller fraction in the root-shoot axis. By the 5th day this proportion had decreased to about one-sixth or less for all tissues except the shoot tips, where the amides constitute almost half the total amino acids. Thus it appears that although asparagine and glutamine are important intermediate compounds, they do not predominate to the extent once believed. Other recent work (21, 24) indicates substantial amide accumulation only in etiolated pea seedlings in later stages of growth.

In any such analytical scheme as used here, some uncertainties exist. The extraction with 70 % alcohol is widely used in plant studies for non-protein nitrogen. Whether the soluble and insoluble peptide fractions might be considered proteinaceous is perhaps a matter of definition. However, it is clear that a very significant quantity of compounds larger than monomeric are soluble in alcohol, and others which are precipitated by alcohol are not precipitated by trichloracetic acid or by acetic acid. In the alcohol-soluble group, about 10 to 25 % may be accounted for by glutathione (14). The remaining peptides, as well as the amino acids and amides, are probably intermediates in the protein transformations. Danielsson (9) has shown that relatively high molecular weight intermediates are present during protein synthesis in the ripening seeds, and has suggested the possibility that the albumin plays this role during germination. In the beginning there is a much larger amount of these peptides present than free amino acids, and throughout the experimental period a substantial quantity is present. It is worth noting that the amount of alcohol-insoluble peptides in the cotyledons falls as the free amino acids, amides and alcohol-soluble peptides in the cotyledons increase, and rises again on the 5th day when the alcohol-soluble nitrogen compounds begin to decrease.

The various tissues of the pea seedling differ in their percentage composition of nitrogenous compounds, as is shown by table III. The root and shoot tips have a very high total nitrogen and protein concentration, and also a relatively high concentration of non-protein nitrogen. The high protein concentration is probably related to the synthesis of new material in the meristematic cells. The non-protein nitrogen level may be higher than it otherwise would have been if it had been possible to separate completely the dividing from non-dividing cells (figs 1 and 2). The root and shoot shafts where the latter type of cells predominate were found to be very high in the non-protein nitrogenous constituents. However, Steward et al (23) found the protein and non-protein nitrogen concentrations to be about the same in shoot apex meristems of 14-day old seedlings of

TABLE III

CONCENTRATION OF NITROGENOUS CONSTITUENTS OF PEA SEEDLINGS *

TYPE OF TISSUE	TOTAL N	PROTEIN N	ALCOHOL-INSOLUBLE PEPTIDE N	ALCOHOL-SOLUBLE N	FREE AMINO N	AMIDE N	AMMONIA N	UREA N	ALCOHOL-SOLUBLE PEPTIDE N
				Pea seeds					
Cotyledons	4.4	3.8	0.26	0.3	0.09	0.031	0.005	0.002	0.03
Root-shoot axis	7.0	5.7	0.48	0.8	0.19	0.036	0.014	0	...
Seed coat	0.7	...	...	0.06	0.016	...	...	...	...
				One-day-old seedlings					
Cotyledons	4.3	3.7	0.22	0.42	0.105	0.04	0.003	0.004	0.03
Root-shoot axis	7.1	5.6	0.40	1.07	0.30	0.06	0.010	0.012	...
				Three-day-old seedlings					
Cotyledons	4.2	3.5	0.14	0.56	0.20	0.04	0.009	0.004	0.07
Shoot tips	8.3	5.9	0.16	2.2	0.85	0.22	0.07	0.03	0.08
Shoot shafts	6.0	3.0	0.19	2.8	1.5	0.27	0.08	0.04	0.14
Root shafts	6.2	2.6	0.14	3.5	2.0	0.32	0.10	0.07	0.13
Root tips	8.7	6.2	0.11	1.9	0.9	0.12	0.06	0 02	...
				Five-day-old seedlings					
Cotyledons	4.3	3.5	0.20	0.6	0.21	0.034	0.008	0.003	0.08
Shoot tips	9.0	6.8	0.16	2.0	0.73	0.33	0.06	0.04	0.12
Shoot shafts	6.2	2.2	0.13	3.9	2.3	0.40	0.08	0.05	0.12
Root shafts	6.4	2.6	0.17	3.6	2.2	0.30	0.10	0.05	0.15
Root tips	7.8	5.8	0.14	1.8	0.9	0.10	0.06	0.02	...

* % based on dry weight.

Lupinus alba. They separated the leaf primordia from the meristems. This was not done in the present work. They found the ratio of protein to non-protein nitrogen to be higher in the leaf primordia. Presumably, differences in age and species in the 2 studies account for the differences found in nitrogen distribution.

In the root and shoot shafts, over half the total nitrogen was found to be alcohol-soluble. If one assumes the average molecular weight (per nitrogen atom) of the compounds in these parts to be 100, such materials would make up roughly one-quarter of the total dry weight. Root and shoot shaft tissue was correspondingly high in free amino nitrogen, amides, ammonia and urea as compared with other tissues. During the 5-day period studied these alcohol soluble constituents increased in absolute amount in their relation to total dry weight, and in relation to the other nitrogen compounds present. The predominance of these materials in the shafts fits in with the concept that these organs are functioning to transport the raw materials for new protein synthesis from the cotyledons to the root and shoot tips.

We have no explanation for the fact that the concentration of amides in the root tips is lower than in all the other tissues except the cotyledons. This is the one class of compounds in which the root tips were found to differ from the shoot tips. No important differences were found between root and shoot shafts.

The cotyledons are lower in total nitrogen than the other tissues. Most of the nitrogen is present as protein, and Danielsson's data (8) indicate that about three-quarters of this is the reserve protein, globulin. However, his data are not directly comparable, since they deal with the protein extractable with buffered dilute NaCl solution. Also, his seedlings grew considerably slower than those in the present work. It is shown in table III that nitrogen compounds in the seed coats are very low. Though urea has been shown to be a not uncommon constituent of plant tissues (7, 18) the amount present in these pea seedlings was too small to have much apparent importance.

A considerable amount of the alcohol-soluble nitrogen remains unaccounted for in all types of sample. This was around 40 % in most cases, but all but about 25 % was accounted for in the root-shoot axis of the 5-day seedling, suggesting that the unknown materials decrease as the plant becomes older. It is not difficult to think of numerous candidates for the vacancies. Significant amounts of adenine (13) and choline (5) have been reported in peas. Pipecolic acid, β-alanine and the imino acids (16) would be under-represented. Other purines and pyrimidines, chlorophyll, and various nitrogen containing B-vitamins are undoubtedly present.

Summary

Peas were germinated in vermiculite at 18° C. and the seedlings grown for 1, 3 and 5 days. The harvested samples were dissected into cotyledons and embryonic root-shoot axis for the initial and 1-day samples; and into cotyledons, root tips, shoot tips, and root and shoot shafts for the 3- and 5-day seedlings. The samples were analyzed for total nitrogen, nitrogen soluble in 70 % ethanol, free amino nitrogen, amides, ammonia, urea, alcohol-soluble peptides and alcohol-insoluble peptides.

From the beginning, the amount of protein in the cotyledons steadily decreases while the protein in the growing parts increases. The non-protein nitrogenous constituents in the cotyledons increase in amount during the first 3 days and then begin to decrease, while in the root-shoot axis these constituents increase slowly at first, and then very rapidly. The pattern suggested is the simple one of nitrogen being mobilized by breakdown of reserve cotyledon to form nitrogenous intermediates in the cotyledons, which are then transported by way of the root and shoot shafts to the sites of synthesis of new protein at the growing apices.

In these young pea seedlings grown under conditions simulating the normal, amides represent only one-sixth to one-half of the free amino nitrogen. Substantial amounts of peptides are present. Root and shoot shaft tissue is characterized by comparatively high concentrations of non-protein nitrogenous compounds, while root and shoot tips are very high in protein.

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BOUND WATER AND FROST HARDINESS[1, 2]

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The suggestion that "bound water" may play a role in frost hardiness has led to much confusion in the past (4). This is partly due to erroneous methods of measurement, partly to misinterpretation of results because of a confusion between osmotically bound and colloidally bound water (4). In recent years, the whole concept of "bound water" has been questioned by some investigators and avoided by others. Yet the fundamental meaning of bound water and its possible role in frost hardiness may actually be temporarily ignored without destroying its usefulness. For, if a sound method of measurement is developed, it may be used as a tool to help isolate the substance or substances whose change during the hardening period is primarily responsible for the development of hardiness.

One fact that does emerge from the bound water controversy is that the quantity can be clearly defined to the satisfaction of all investigators only on the basis of the method used to measure it. Since so many different methods have been used, it is obvious that many definitions will emerge. The fraction of the total water that is taken as bound water will, of course, vary with the method used. Since the problem undertaken in this investigation was to determine whether hardening increases the hydration capacity of protoplasm or one of its constituents (at any 1 vapor pressure) it does not matter what fraction of the total water is taken as bound, provided that the same method is used in comparing hardy and nonhardy tissues. By definition, bound water will be that fraction not removed by the force used experimentally to remove water. In this investigation, it is the fraction not removed when in equilibrium with $Mg(ClO_4)_2$ at 0 to 5° C, but removed at 105° C. Conversely "free water" in this investigation will refer to water removed by this force.

Distinction between "osmotically bound" and "colloidally bound" water is made in spite of the fact that no difference exists in the nature of the water bound. The sole purpose of distinguishing between the 2 is in order to discover which cell components are responsible for the effect.

There is now sufficient evidence to postulate that the increased hardiness as a result of the accumula-

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tion of sugars during hardening, is due to an osmotic effect (4, 5, 6). But it is an indisputable fact that sugars cannot account for the whole increase in hardiness; in fact, in some plants they are unable to account for any of the increase (4). In other words, some substance in the protoplasm undergoes a change during the hardening period that results in the major, if not the sole increase in frost hardiness. Since this protoplasmal change seems to be associated with a change in hydration (4), bound water measurements may conceivably be used in identifying and isolating the hydrated substance.

Previous work (6) showed that evacuation at room temperature (or at 3° C) at a relative humidity of essentially 0 %, does not remove all the water from the mycelium of *Aspergillus niger*. More water is removed by heating to 80 to 110° C. This latter weight loss was defined as bound water. The amount present was found to vary directly with the hardiness of the mycelium. Later work showed that this method could also be applied to measure the bound water of protoplasmal fractions (3). Füchtbauer (1) failed to find any relation between drought hardiness and bound water of yeast by this method. But he failed to grasp the basic principles of the method and therefore applied it incorrectly. Instead of 0 % relative humidity, he used considerably higher values; and instead of drying for a minimum of a month, he dried his samples only for several hours. The aims of these 2 precautions are a) to remove all the osmotically bound water during the 1st drying without removing all the colloidally bound water, and b) to allow sufficient time for driving off all or nearly all the colloidally bound water during the 2nd drying.

Methods

a) Determinations of Bound Water: One to two month seedlings of cabbage (*Brassica oleracea capitata* var. Early Jersey Wakefield) were frost hardened for from 1 week to 2 months in the usual way by transferring to a cold room (+3° C) under a 200 watt lamp. The light was automatically set to be on 12 hrs a day. The leaves of hardened and unhardened plants were 1st freeze-dried as follows. They were frozen quickly (within 5 minutes) by placing them on the floor of a deepfreeze at −20° C. They were then transferred to a desiccator containing dry Al_2O_3 that had been allowed to come to equilibrium with the deepfreeze temperature. In order to overcome the resistance to water movement offered by the cuticle, each frozen leaf was broken into 3 or 4 pieces. The desiccator was then evacuated and left in the deepfreeze for 5 to 7 days, after which it was allowed to come to room temperature. The dry leaves were then removed and either transferred directly to weighing bottles or ground in a Wiley mill, depending on the experiment. Before weighing, the samples were first allowed to come to equilibrium with 0 % relative humidity by evacuating over $Mg(ClO_4)_2$ in a desiccator kept at 3° C. An Abbeon relative humidity indicator registered below zero in this desiccator. The samples were weighed at weekly intervals (first allowing the desiccator to come to room temperature) until a constant weight was reached. The 2nd drying of the weighed samples occurred in an oven at 103° C. Weighings were again made at weekly intervals.

b) Fractionation of Leaf Powder: In preliminary tests an attempt was made simply to separate the soluble from the insoluble components. For this purpose, 25 ml distilled water was added per gram powder (in 1 case half as much water was used—see discussion). After thorough mixing the extract was filtered through a Buchner funnel in a cold room at 3° C. The extract and the residue were then separately freeze-dried. In later tests, the protoplasmal components were separated by the method described earlier (2). This enabled the separation of 6 fractions: 2 particulate fractions, acid insoluble proteins, globulins, albumins and residue. In the case of potato tubers, the 2 particulate fractions are usually called mitochondria and microsomes, respectively. In the case of leaves, this terminology is unsatisfactory since although the whole chloroplasts are thrown down by the 1st centrifuging, the mitochondrial fraction is also green. Observation under the microscope revealed that this color is due to the presence of green grana. Even the microsome fraction contained a small amount of these grana.

In the 1st experiment, the 2nd drying was allowed to continue at 103° C for 2 months. Dry weights taken every 2 to 3 days for about 2 weeks agreed closely enough as shown by the standard deviation (table I). But in spite of this small standard deviation, later tests showed that the samples continued to lose weight slowly if left long enough (table II), even for as long as 7 months. Nevertheless, the bound water content was higher in the hardened than in the unhardened samples whether drying proceeded for 1 week or for 7 months. The value for the unhardened samples was of the same order as in the

Table I

Bound Water in Hardened and Unhardened Cabbage *

Treatment		Wt after 1st drying (mg)	Loss during 2nd drying (mg)	% bound water (2nd loss as % of 1st dry wt)
Unhardened	1	76.25 ± 0.04	7.3 ± 0.25	9.6
	2	69.25 ± 0.23	7.4 ± 0.025	10.7
Hardened 8 days	1	57.43 ± 0.02	6.8 ± 0.09	11.9
	2	38.47 ± 0.08	4.6 ± 0.02	12.0

* First drying by evacuation over $Mg(ClO_4)_2$ at 3° C; 2nd at 103° C for 2 months. The standard deviation is given in each case.

TABLE II

BOUND WATER IN 4-WEEK HARDENED AND UNHARDENED CABBAGE IN RELATION TO TIME ALLOWED FOR 2ND DRYING*

	UNHARDENED			HARDENED				
TIME AT 103° C	1ST DRY WT (MG)	LOSS AT 103° C (MG)	% BOUND WATER	1ST DRY WT (MG)	LOSS AT 103° C (MG)	% BOUND WATER	BW/BW	H/NH
	159.15 ± 0.06			308.35 ± 0.14				
1 week		9.2	5.8		42.3	13.7	2.4	
2 "		12.2	7.7		49.2	15.9	2.1	
3 "		12.2	7.7		51.9	16.8	2.2	
4 "		13.2	8.3		55.1	17.9	2.2	
2 months		15.4	9.7		59.6	19.3	2.0	
6 "		18.8	11.8		69.7	22.6	1.9	
7 "		20.0	12.6		71.7	23.2	1.8	

* First drying by evacuation over $Mg(ClO_4)_2$ at 3° C, 2nd at 105° C.

1st experiment if compared after the same drying period.

The ratio of bound water in the hardened to that in the unhardened dropped slightly with the time allowed for the 2nd drying. It must be realized, however, that the loss in weight at 103° C may not be due solely to water loss. Other volatile substances may also be given off. There is some evidence for this; preliminary tests showed that leaves of 2 succulents (*Sempervivum glaucum* and *Saxifraga cordifolia*) continue to lose weight over a period of several months at a greater rate than the cabbage leaves. This is possibly due to a much higher organic acid content in the succulents, some of which may slowly volatilize. In the same way, the fact that unhardened cabbage is slightly more acid than hardened cabbage (4) may perhaps explain the slight decrease in the ratio of bound water in the hardened to that in the unhardened material. This may be due to the predominant loss of water at first and the proportionately greater loss of volatile organic acid during later stages of drying. If this is true, shorter drying periods at 103° C may give more reliable bound water ratios. But it must be emphasized that the 1st drying period cannot be shortened without invalidating the results. This drying must continue long enough (depending on the size of the sample) for all the free water to be removed, a period of about 2 months in the case of larger samples.

It is still conceivable that water of hydration of sucrose might be involved, since the sugar content is higher in hardened leaves (4). The attempt was therefore made to separate the soluble from the insoluble components. In order to do this, the leaves were first ground. A control sample of the ground leaves was also tested to make sure that the grinding process itself did not alter the results.

Table III shows that the grinding process fails to alter the results. The leaf powder from the hardened plants showed the same increase in bound water over that from the unhardened plants as when unground leaves were used. Both the extract and the insoluble residue also showed differences in the same direction, though the amount of bound water was much less in the latter. In view of this fact, it might even be suggested that the bound water in the residue is due to unextracted solutes. In agreement with this suggestion is the fact that the bound water in the residue from the hardened leaves is at least 70 % greater in the 1st experiment and therefore more solutes must have been left unextracted.

Since the excess bound water in the hardened leaves is mainly found in the soluble extract, and since this soluble component is known to consist mainly of sugars, the higher bound water content of the hardened leaves could be logically explained by their higher sugar content. In order for bound water determinations to give any information about the protoplasmic components, it is therefore necessary to obtain these free of sugars and other soluble substances (table IV).

Of the 5 protoplasmic fractions, the only 1 that

TABLE III

PERCENT BOUND WATER IN SOLUBLE AND INSOLUBLE FRACTIONS OF CABBAGE LEAF POWDER

LEAF NO.	HARDENING TREATMENT (TIME AT 3° C)	DRYING TIME AT 103° C (MONTHS)	LEAF POWDER		LEAF EXTRACT		LEAF RESIDUE	
			UNHARDENED	HARDENED	UNHARDENED	HARDENED	UNHARDENED	HARDENED
7–11	3 weeks	1	10.0	18.0	15.5	18.5	3.9	12.4
		2	11.0	19.5	17.3	25.1	5.3	14.1
		3	13.2	22.4	18.4	26.4	8.2	17.3
		4	14.9	24.7			10.3	19.8
5–10	2 months	1	9.9	17.2	13.6	25.5	4.0	5.4
		2	10.7	19.2	16.7	31.3	5.3	6.7
		3	13.4	22.2	18.4	32.0	8.7	9.9
		4	15.8	23.6			11.2	12.9
12–15	2 weeks	1	8.0	18.9	13.9	25.0	2.7	4.6
		2	9.0	20.5	17.4	31.0	3.8	6.2
		3	11.0	22.6	18.7	32.5	6.1	9.0
		4	12.6	24.4			8.5	11.9

TABLE IV

PERCENT BOUND WATER IN PROTOPLASMIC FRACTIONS OF HARDENED AND UNHARDENED CABBAGE LEAVES*

FRACTION	UNHARDENED LEAVES		HARDENED LEAVES	
SAMPLE	1	2	1	2
Mitochondria (plus grana)	16.5	18.1	19.2	20.4
Microsomes (plus grana)	21.1	26.5	25.0	22.2
Acid insoluble proteins	10.7	5.7	9.2	8.7
Globulins	5.6	7.0	12.3	6.4
Albumins	12.0	12.4	14.3	9.9
Original leaf powder		9.5		12.2

* Dried 2 to 3 months at 103° C.

showed a higher bound water content in the hardened leaves of both experiments was the mitochondria (plus grana). In view of the contamination of this fraction with chloroplast grana, and also because chloroplasts are known to look different in hardened than in unhardened cells (4), these protoplasmic particles were next investigated.

The separation of chloroplasts from leaf homogenate is usually done in a sucrose solution to prevent osmotic rupture and consequent release of the grana. In these experiments, however, it was necessary to avoid adding sugar in order not to introduce a substance that is itself capable of binding water. The same precaution was necessary in the case of the other protoplasmic particulates. Ammonium acetate (plus a small amount of ammonium hydroxide to neutralize the cell sap) was, therefore, again used. It is true that the chloroplasts do not retain their normal state as well in this medium as in sugar solutions, but there is no reason for expecting it to alter the bound water content. It has another advantage over sugar solutions—the particles are more readily centrifuged down in this medium, presumably because of its lower specific gravity.

Since the chloroplasts do contain starch, and to a larger degree in the unhardened plants, it was feared that this might markedly alter the results, either due to binding of water by the starch, or to a "dilution" of the remaining chloroplast constituents. Consequently after centrifuging down the chloroplasts in 1 experiment they were allowed to rupture in distilled water, and the released starch grains were 1st centrifuged down at a higher speed. Thus, both whole chloroplasts and grana were separately collected from both unhardened and hardened leaves for bound water determinations.

In all cases, the whole chloroplasts and grana from the hardened leaves had higher bound water contents (table V).

TABLE V

PERCENT BOUND WATER IN CHLOROPLASTS AND GRANA OF HARDENED AND UNHARDENED CABBAGE LEAVES*

EXPERIMENT	FRACTION	% BOUND WATER REMOVED BY DRYING AT 105° C			
		DRIED FOR 2 WKS.		DRIED FOR 5 WKS.	
		UNHARDENED	HARDENED	UNHARDENED	HARDENED
1	Chloroplasts	5.5	7.3	7.2	9.7
2	Chloroplasts	11.4	14.3	12.7	15.5
		9.8	11.8	11.7	14.3
3	Grana	4.1	14.0	6.1	14.0

* In 1, 3g leaf powder were extracted with 48 ml. 0.2 M NH_4 acetate; in 2 and 3, 0.5g were extracted with 50 ml.

SUMMARY

1. Leaves of hardened cabbage dried at 0 % relative humidity retained larger amounts of bound water than leaves of unhardened cabbage.

2. Both leaf extract and leaf residues showed the same differences as the whole leaf.

3. The mixture of mitochondria and chloroplast grana, the whole chloroplasts, and the grana all showed the above differences; the cytoplasmic proteins and the mixture of microsomes and grana did not.

4. The method adopted for measuring bound water therefore succeeded in showing that at least some protoplasmic components undergo an increase in hydration capacity during the hardening period.

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News and Notes

Announcement of Travel Grants At the request of fund-granting agencies and with the advice of appropriate scientific societies, the Division of Biology and Agriculture of the National Academy of Sciences—National Research Council is establishing screening committees to allocate pooled funds to applicants for travel grants to the following international congresses:

Photobiology, Copenhagen, July 31 to August 5, 1960
Histochemistry, Paris, August 29 to September 1, 1960
Cell Biology, Paris, September 3 to 8, 1960

Send requests for travel grant application forms to the Division of Biology and Agriculture, NAS-NRC, 2101 Constitution Avenue, Washington 25, D. C. Awards will be in an amount roughly equivalent to round trip air fare to the congress. Completed application forms *must* be in the Division office not later than January 1, 1960. Awards will be announced by February 1, 1960.

Travel Grants to Grassland Congress The National Science Foundation will award grants to defray partial travel expenses for a limited number of U.S. scientists who wish to participate in the Eighth International Grassland Congress. This congress will be held in Reading, England, July 11 to 21, 1960.

Obtain application blanks from the National Science Foundation, Washington 25, D.C. *Completed* application forms must be submitted by February 1, 1960.

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